NOTE TO USERS

THE ACTIVATION OF ENDOGENOUS NEURAL PRECURSORS IN THE ADULT RODENT EYE

by

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DEDICATION

For my family...without question.

The truly happy individual can recognize and pursue opportunity in all of its forms...including those that embrace the expansion of mind, being and love. The truly successful individual realizes that no single one of these will lead them to their "dream life". The tricky part, then, is finding the fulcrum which permits that delicate balance. I'd like to think that I achieved that balance, in all of its forms during my PhD. If nothing else, that very fact gives me a sense of pride and distinction.

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ABSTRACT

The loss of neurons in the central nervous system (CNS) is accompanied by a range of pathophenotypes, ranging from mild to profound functional deficits. The discovery of cells within the CNS that are capable of producing new neurons, however, has spawned a litany of interest in "adult neural precursor" science. It is clear that adult neural precursors respond to CNS pathology, and increase their production of neurons when treated with selected factors and genes that are amenable to neurogenesis. In the adult mammalian retina, *bonafide* retinal stem/progenitor cells reside in a quiescent state. Furthermore, the mammalian eye provides a convenient and well studied model to investigate CNS injury and cell replacement treatments. In this thesis, I investigated the effects of selective neuronal injury, as well as the exogenous application of mitogenic growth factors, on the activation of cells in the eye that bear functional and phenotypic resemblance to retinal progenitor cells (RPCs). Induction of retinal ganglion cell (RGC) death resulted in a proliferative response, the up-regulation of transcriptional and cytoskeletal phenotypes akin to those seen in RPCs, and the emergence of a population of cells in the ciliary body (CB) resembling retinal neurons (Chapter 2). When exposed to intravitreally administered epidermal growth factor (EGF) and/or erythropoietin (EPO), Müller glia, the principal glial cell of the retina and neural precursor in lower-vertebrate animals, display RPC-like behaviors ranging from proliferation to expression of transcriptional machinery involved in progenitor function (Chapter 3). Finally, the isolation of Müller glia and maintenance in culture in the absence of retinal neurons permits these cells to exhibit hallmark features of neural precursors, including the formation of sphere-like aggregates, the up-regulation of pro-neural phenotypes, and production of cells that express neuronal phenotypes when exposed to growth factors (Chapter 4). Collectively, these data indicate that the adult mammalian eye contains neural precursors, including those derived from mature Müller glia, that respond to injury and exogenous growth factor treatment, and retain functional and phenotypic characteristics of RPCs.

LIST OF ABBREVIATIONS USED

ANOVA	Analysis of variance
BDNF	Brain-derived neurotrophic factor
bHLH	basic helix-loop-helix
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CB	Ciliary body
CE	Ciliary epithelium
CM	Ciliary margin
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
DIC	Differential interference contrast
DMEM	Dulbecco's Modified Eagle's Medium
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EPO	Erythropoietin
EPO-R	Erythropoietin receptor
bFGF	basic fibroblast growth factor
FGF-2	acidic fibroblast growth factor
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
GLAST	Astrocyte-specific glutamate transporter
GS	Gutamine synthetase
HD	Homeodomain
HIF-1α	Hypoxia inducible factor 1α
HRE	Hypoxia response element
Ig	Immunoglobulin
ILM	Inner limiting membrane
INL	Inner nuclear layer
IPL	Inner plexiform layer

LIF	Leukemia inhibitory factor
MAPK	MEK/mitogen-activated protein kinase
NFL	Nerve fiber layer
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartic acid
NPC	Neural precursor cell
OLM	Outer limiting membrane
ON	Optic nerve
ONL	Outer nuclear layer
OPL	Outer plexiform layer
P	Postnatal day
PB	Phosphate buffer
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PLC	Phospholipase C
RA	Retinoic acid
RGC	Retinal ganglion cell
RPC	Retinal progenitor cell
RPE	Retinal pigmented epithelium
Rt	Room temperature
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
SEM	Standard error of the mean
Shh	sonic hedgehog
SVZ	Subventricular zone
TF	Transcription factor
Trk	Receptor tyrosine kinase
TrkB	Receptor tyrosine kinase B

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In pursuit of distinction, I quickly realized that the road to success in this domain, as with many endeavors, becomes much wider, smoother and easier to navigate when in the presence of great people. Mentors, technical advisors, smiling faces, and in my mind the most important, family and good friends, make the challenge surmountable.

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CHAPTER 1: Introduction

The Retina

Retinal Anatomy and Visual Processing: An Overview

Retinal Anatomy

The adult retina is a multilayer, neural structure contained within the posterior aspect of the globe of the eye. Although the retina contains 7 main classes of neural cells, it also contains endothelial cells associated with vasculature, non-retinal astrocytes and peripherally derived, phagocytotic microglia and an epithelium (See "The retina: an approachable part of the brain", John Dowling, Belknap Press, 1987). As depicted in Figure 1-1, retinal cells reside stereotypically within multiple laminae, which themselves contain either cell bodies (nuclear layers) or neuronal processes (plexiform layers). These layers are arranged along the vitreo-scleral axis as follows: inner limiting membrane (ILM - a barrier comprised of Müller glial end feet); nerve fiber layer (NFL - containing retinal ganglion cell axons and astrocytes); ganglion cell layer (GCL - containing retinal ganglion cell (RGC) bodies and displaced amacrine cells); inner plexiform layer (IPL), containing processes of RGCs, bipolar cells and amacrine cells); inner nuclear layer (INL), containing amacrine, bipolar, Müller and horizontal cell bodies; the outer nuclear layer (ONL), containing rods and cones; outer limiting membrane (OLM), which is the outermost barrier formed by Müller glial apical processes; a photoreceptor layer (PR) containing the outer segments of rods and cones; and finally, the retinal pigmented epithelium (RPE), a support structure which resides posterior and juxtaposed to the photoreceptor outer segments.

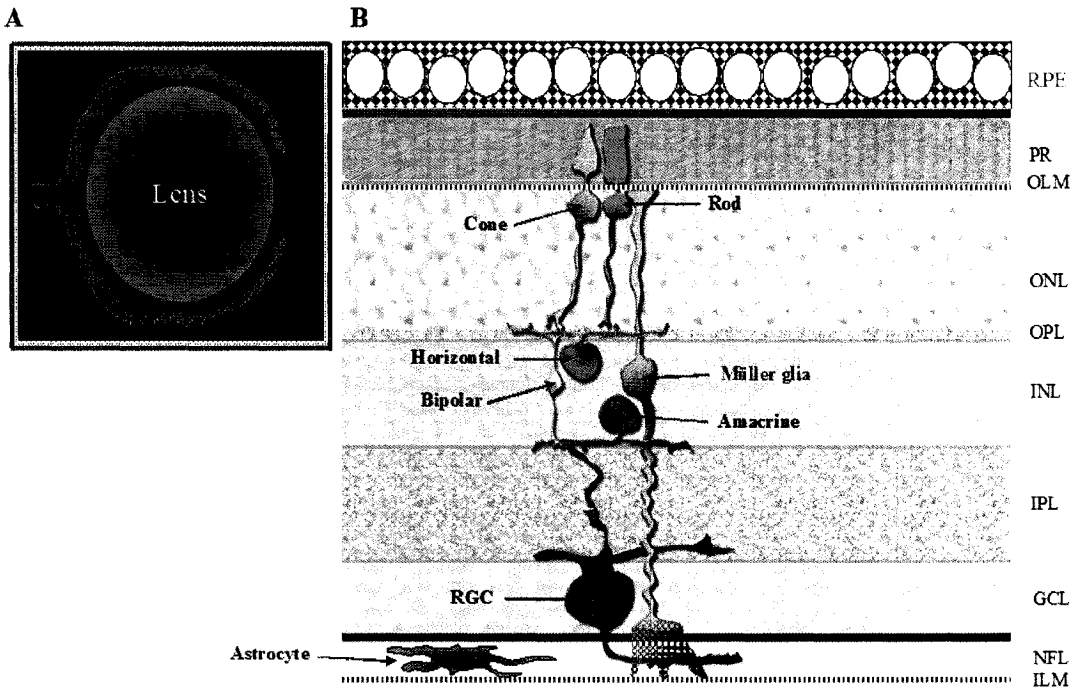


Figure 1-1. Microanatomy of the mammalian retina. **(A)** This diagram of a sagittal section through the eye shows that the retina (blue) resides on the posterior aspect of the globe. **(B)** The retina contains 6 classes of neurons (retinal ganglion cells (RGC), amacrine cells, bipolar neurons, horizontal cells, rods and cones) as well as a class of glia termed the Müller cell. These cells reside in nuclear layers that are separated by plexiform layers, which themselves are comprised of neuronal processes. The vitreo-scleral boundaries of the neural retina are the inner (ILM) and outer (OLM) limiting membranes formed by Müller cell endfeet and apical processes respectively. The retinal pigmented epithelium (RPE) lies juxtaposed, and deep to the neural retina. A class of astrocyte also resides in the nerve fiber layer (NFL), and is derived from the optic nerve during development. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; OLN, outer nuclear layer; OLM, outer limiting membrane; PR, photoreceptors.

Although the retina contains 7 main classes of cells, it is thought that as many as 55 different cell types can be identified based on morphological, electrophysiological and neurochemical criteria (Masland, 2001). This impressive variety of distinct cell types is largely conserved across vertebrate species, and is typical of the cytodiversity seen in other, discrete regions of the CNS. It is clear that the high demands of visual processing require a variety of specific cell types in order to function at an optimal level, and that this cellular diversity has evolved through an evolutionarily conserved mechanism (Fernald, 2004).

Visual Processing

Light enters the eye through the cornea, travels through the aqueous humor in the anterior chamber, enters the lens where it is refracted and projected through the vitreous humor onto the neural retina. As light enters the retina, it must pass through non-retinal astrocytes, three nuclear and two plexiform layers until it is absorbed by phototransducive pigments located in the outer segments of photoreceptors. Refracted and residual light is absorbed by retinal pigmented epithelial (RPE) cells located deep to the neural retina. This absorptive property is presumed to function in reducing both light-induced damage to photoreceptors, and refractive activation of photoreceptors that are positioned adjacent to the focus of illumination. Following illumination of PR outer segments, light energy is then transduced into a neural signal by resident photoactive pigments, and is processed locally within the retina via communication through a series of interneurons to the ganglion cell. Signaling proceeds via a vertical pathway (photoreceptor - bipolar cell – RGC). At the retinal level, visual information pertaining to movement and contrast is achieved via lateral processing provided by horizontal and

amacrine cells. Information converges on RGCs where it exits the eye via the optic nerve (ON), which itself is comprised of RGC axons. The ON becomes the optic tract caudal to the optic chiasm, and subsequently projects to a number of hindbrain, midbrain and forebrain regions responsible for vision, blinking and biological rhythms (for review, see Kandel, Schwartz and Jessell, 4th edition). In the rodent, 95% of RGC axons decussate at the optic chiasm whereas only 50% do in the human. In addition, RGCs project primarily to the superior colliculus in the rodent, which is in contrast to human where they terminate primarily in the lateral geniculate nucleus.

The Retina as a Model of CNS Development

The retina is a structure belonging to the CNS, and has been the focus of intense research in the field of developmental neurobiology. The mechanisms which govern the proliferation of retinal precursors, and cellular diversity generated throughout retinal development, are presently among the most widely understood of all CNS systems. The allure of the retina as a focus of research is partly due to its accessibility. The retina resides extracranially within the oculus, which provides convenient access without having to perform highly intrusive intracranial surgery. Early studies aimed at characterizing the timing and source of retinal cell production demanded that intraocular injections of viral lineage tracing constructs were performed during early post-natal times (Turner and Cepko, 1987). The development of accurate surgical methodology for intraocular injections was benefited by the fact that the eyes of newborn pups and adults are covered by a thin lid membrane, which in turn, permits easy access to the eye. Additional benefits arise when one attempts to analyze the retina histologically. The microanatomy of the retina is arranged histotypically (predominantly the laminar

distribution of distinct cell types and their processes) which permits the facile identification of single classes of retinal cells. Although this stereotypic distribution of cells is present to a lesser extent in hippocampus, cortex and spinal cord, the high fidelity of the retinal histotypical arrangement is not seen in many other areas of the CNS. We can assert, for example, that cells residing in the ONL of the retina are photoreceptors, which simplifies our efforts to dissect out that particular cell of interest.

The impact of any discovery made in developmental neurobiology hinges greatly on the degree to which the findings explain phenomena across a number of different structures and developmental stages. The retina is a CNS structure that can be regarded as an extension of the brain, which itself receives afferent input from RGCs located within the eye. The retina is derived from the neuroectoderm, and retains many features akin to those seen in both the brain and spinal cord. It is clear that research in retinal science, such as that found in the fields of neural development and cell survival, has contributed to our understanding of congruent developmental mechanisms functioning in brain and spinal cord (reviewed by Harvey et al., 2006), which themselves are also of neuroectodermal origin. As will be discussed, the transcriptional mechanisms that regulate proliferation and fate determination within the developing neural tube are also present in the developing retina (Figure 1-2) highlighting the degree to which these mechanisms are conserved throughout organogenesis. Thus, a concept emerges with which structures can be compared to one another, and can be done so heterochromatically (from different developmental times). To extend this notion even further, the retina exhibits an impressive conservation of these same developmental mechanisms across a variety of species. Very few structures retain the same degree of evolutionarily conserved

order of cell genesis, and molecular determinants of cell fate (Marquardt, 2003). As a consequence, the use of rodents has been proven to be indispensable for understanding basic determinants of cell fate and organogenesis, as well as the development of treatments amenable to alleviating human retinal and non-retinal CNS pathologies.

Retinogenesis

Developmental Origin of the Retina

The retina is a multilaminar, neural structure whose function is to transduce and process photic information from the external environment, and transmit that information to the brain. This process involves the co-coordinated effort of highly specialized cell types, each of which must be produced, subsequently migrate to an appropriate stereotopic position, differentiate into a fully functional post-mitotic cell, and form connections with appropriate cohort cells. Early lineage tracing experiments determined that the production of all 7 main cell classes (retinal ganglion, amacrine, bipolar, horizontal, rod, cone and Müller glial cells) within the neural retina proceeds in an evolutionarily conserved, histogenic order (Young, 1985).

The cells of the neural retina are produced by stem cells whose origin, in some species, can be traced back to the 16 cell gastrula stage (for review, see Zaghloul et al., 2005). In mammals, the earliest appearance of cells that will give rise to the neural retina is during neurulation, at which time the optic primordia emerge in the ventral midline of the developing anterior neural tube (Hilfer and Yang, 1980). This population of retinal precursors resolves into two distinct eye fields on the prosencephalon that will form the left and right retinae, a process which involves the down-regulation of midline Pax6 expression signaled from prechordal mesoderm (Li et al., 1997). The first gross

morphological evidence of eye formation is the evagination of cells on the surface of the developing diencephalon, which generates bilateral structures termed the optic vesicles (Figure 1-2). Cues from the surface ectoderm signal a bilayer of epithelia residing on the surface of the optic vesicles to invaginate, and form the optic cup. This cup-like structure is continuous with the optic stalk (or presumptive optic nerve) and forms a structure that resembles a rudimentary eye. Contained within the optic cup are two juxtaposed populations of epithelia that will give rise to both the neural retina, and the retinal pigmented epithelium. Cells in the more superficial or outer position relative to the optic stalk give rise to the neural retina, whereas cells residing deep to these give rise to RPE. By E10.5 in mouse, the development of the oculum has manifested into a structure that resembles a rudimentary eye. At this time, RPE and neural retinal progenitors have resolved their respective cytogenic roles to those which support the development of the RPE and retina respectively, corresponding to the initiation of retinal histogenesis (below).

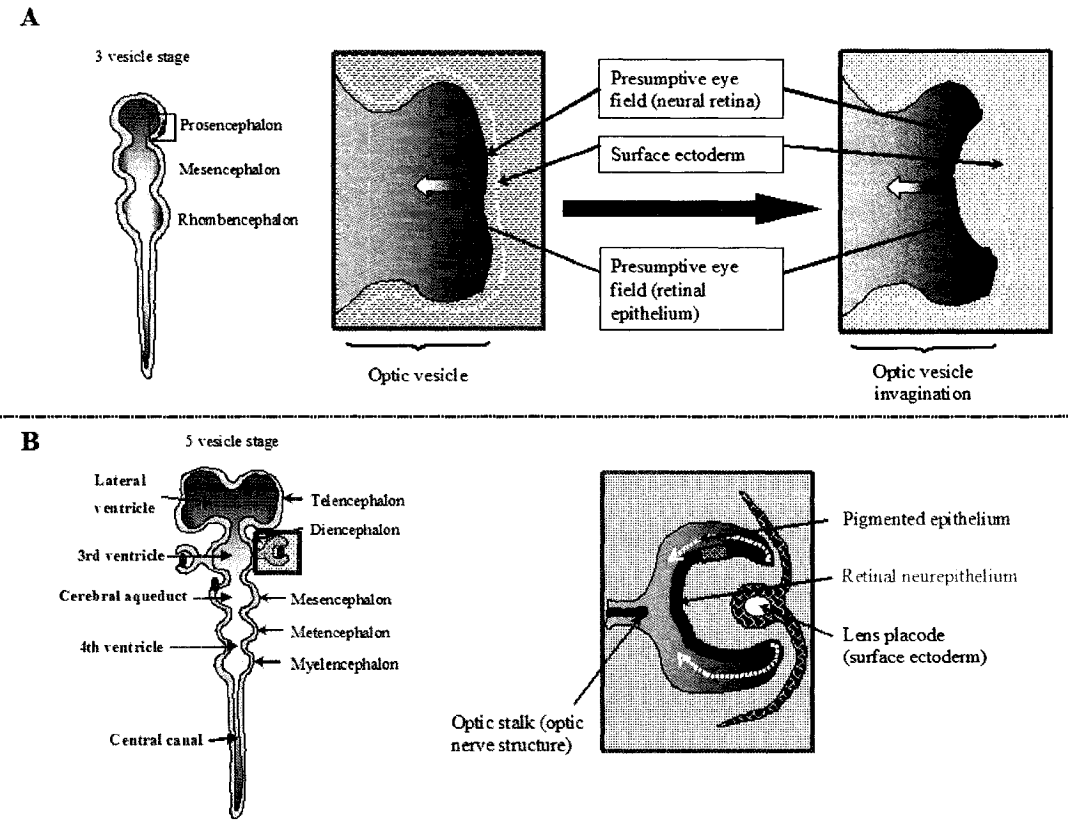
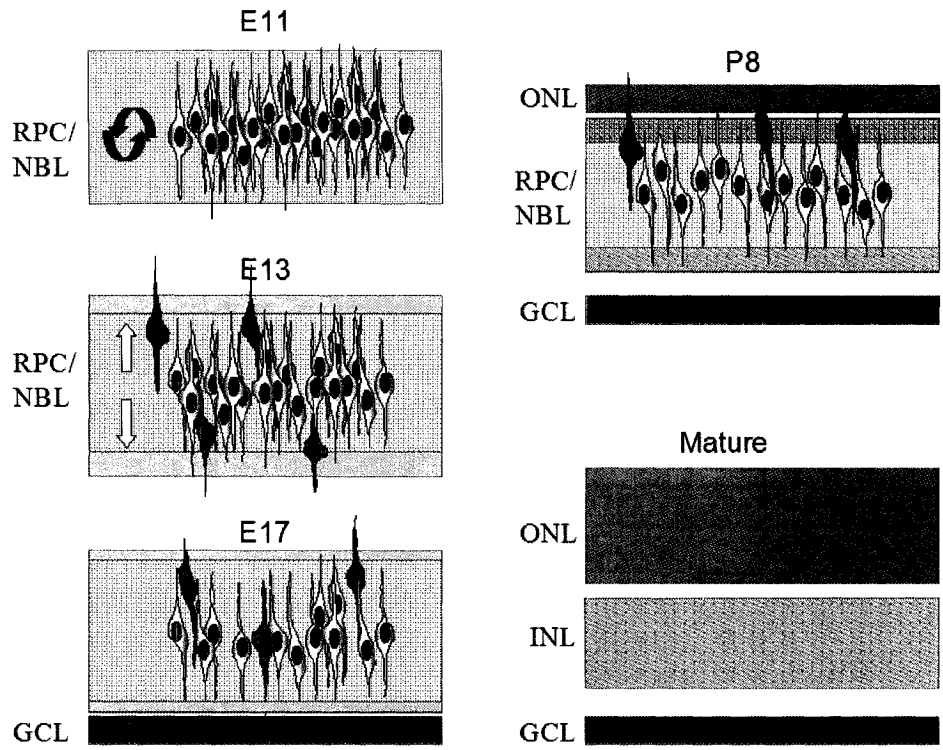


Figure 1-2. Development of the retinal primordia. **(A)** The early precursors to retinal development emerge on the surface of the prosencephalon, which itself will give rise to the diencephalic and telencephalic structures. The surface of the diencephalon develops an outpouching that will form the optic vesicle, which itself invaginates to generate a rudimentary eye cup. **(B)** Bilateral optic primordia resembling eyes reside on the surface of the diencephalon, and are comprised of the optic stalk, RPE and neural retinal epithelia, and the surface ectoderm that gives rise to the lens.

Retinal Histogenesis

During the few days preceding E10.5 in mice, RPCs exist as a pseudostratified layer of neuroblastic epithelium (Figure 1-3). RPCs undergo an aggressive phase of proliferation, during which the clonal division of these cells increases their number to that which is sufficient to generate an appropriate number of cells required for retinal function. The initial RPC number is directly linked to the number and proportion of cells that remain following retinal and cortical development (Levine and Green, 2004; Quinn et al., 2007). This observation underscores the importance of signaling which governs the number and timing of NPCs and RPCs exiting the cell cycle. Following the induction of lens formation within the overlying surface ectoderm, components of the neuroblastic layer (or presumptive neural retina) switch from a self-renewing, symmetrically proliferating state, to one which involves the production of immature, postmitotic retinal cells.

In the earliest phases of postmitotic retinal cell genesis (approximately E13 in mouse), RGCs are produced, followed by the sequential initiation of horizontal, cone and the majority of amacrine cell genesis. These "early" cell types are derived from multipotent RPCs whose propensity toward cell production is biased toward these early cell types. A "later" cohort of cell production (rods, bipolar and Müller glia) follows, and are derived from "late" RPCs (Belliveau et al., 2000). This process of cell production and migration into distinct laminae progresses until P10, at which time all proliferation and retinal cell genesis is complete.



Adapted from Marquardt & Gruss, 2002

Figure 1-3. Development of the mammalian retina: a temporal summary. The E11 mouse retina contains a population of multipotent cells (yellow) that undergo a high rate of proliferation. By E13, transient amplifying cells begin to generate postmitotic precursor cells (red for GCL cells, green for INL cells and blue for ONL cells) that begin to migrate toward their respective target layer. The first cell type produced, the RGC, migrates to the basal surface of the developing retina, and differentiates. By P10, the retina is devoid of proliferation and contains a full complement of postmitotic retinal neurons and glia. The onset of genesis of distinct retinal neuron subtypes ensues, each of which migrate to their final position, thus forming the three nuclear layers of the mature retina.

Pioneering studies which characterized the development of the retina employed the use of H-thymidine and H-thymidine analogues, combined with immunohistochemical phenotyping to date the birth of specific classes of retinal cells (Young, 1985). More advanced lineage tracing studies have permitted us to conclude that the developing retina contains, and is generated by, multipotent RPCs (Cepko et al., 1998; Turner and Cepko, 1987). These studies, spearheaded by the work of Constance Cepko and colleagues, employed the use of replication-incompetent retrovirus constructs that, when exposed to the retinal environment in specific titrations, allow for the identification of single proliferating RPCs and their progeny. Phenotyping of clone populations produced by single RPCs indicated that these cells were capable of generating all 7 classes of neural retinal cells (Turner and Cepko, 1987).

Retinal Progenitor Cell Competence

Despite the successful characterization of the temporal progression of retinal cell genesis, the specific mechanisms which govern the diversity and fidelity by which the enormous variety of specific sub-classes of retinal cells are produced was, and still is, largely unknown. Our understanding of RPC activity, however, has evolved considerably since the mid-1990s. Studies in lower vertebrates demonstrated that diffusible factors were keystone in determining gross ocular patterning during very early stages of development (for reviews see Cepko, 1999; Chow and Lang, 2001). It was apparent, however, that extrinsic, cell non-autonomous cues provided by the surrounding microenvironment were not sufficient to account for the determination of cell fate. It was speculated that these extrinsic factors were working in concert, either antagonistically or synergistically, with intrinsic mechanisms which restrict the fate of cells produced by

single RPCs (reviewed below). One central theory aimed at explaining RPC behavior proposes that RPCs experience temporally choreographed states of competence to give rise to particular retinal cell types. This progressive occupation of "competence states" is superimposed over changing cell cycle dynamics, which themselves include coordinated symmetrical and asymmetrical cell divisions. These "competence states" and cell cycle dynamics are governed by the interplay between the aforementioned intrinsic and extrinsic cues (for review, see Livesey and Cepko, 2001b). Heterochronic transplantation of RPCs, a process in which progenitors are transplanted into a different developmental stage than that which they were derived, elucidated this synergy between intrinsic and extrinsic determinants of cell fate. Specifically, it was determined that extrinsic factors such as ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) were capable of re-directing the propensity of RPCs to produce rods in favor of bipolar cells (Ezzeddine et al., 1997), revealing the important influences that extracellular cues play in fate determination. However, it was clear that RPCs assayed from specific stages of development varied in their propensity toward the genesis of different classes of retinal cells. RGCs, for example, are not produced by "late" progenitors (mentioned earlier), and Müller glia are not produced by "early" progenitors. These observations consolidated the idea that RPCs retain intrinsic limitations in competence that change over the period of development. Brief reviews of both of these classes of factors (extrinsic and intrinsic) are presented below.

The Role(s) of Transcription Factors in Fate Determination and Cell Cycle Dynamics During Retinal Development

During the early 1990s it was evident that two specific classes of transcription factors (TF), those belonging to the basic helix-loop-helix (bHLH) and homeodomain (HD) families, exhibit a high degree of involvement in mediating the cell intrinsic determination of cell fate and cell cycle dynamics (for review see Cepko, 1999). By definition, TFs act as regulators of gene expression by binding to promoter and enhancer sequence elements on DNA, and promoting the recruitment of RNA polymerase complexes (Oxford Dictionary online). During early CNS development, formation of the neural plate is governed, in part, by the expression of multiple TFs (for review, see Edlund and Jessell, 1999). A subset of these factors, namely Pax6, Rx1, Six3, Chx10 and Hes1, are co-expressed in retinal progenitors residing in the optic primordia of the anterior neural plate following neurulation. This profile of TF expression is common to RPCs examined across a number of vertebrate species. Early studies elucidated the importance of TF expression when it was revealed that Pax6, a bHLH TF, is not only necessary, but is sufficient to initiate the formation of rudimentary eye primordia when ectopically expressed is *Drosophila* (reviewed by Chow and Lang, 2001). In vertebrates, knockdown of Pax6 expression results in gross ocular deficit phenotypes ranging from microopthalmia (small eyes) to aniridia (total absence of eyes), as well as a near total reduction in the presence of one retinal cell type, the amacrine cell (for review, see Ashery-Padan and Gruss, 2001). In addition, misexpression studies indicate that Pax6 is also involved in layer specification within the retina (Inoue et al., 2002). This series of observations outline the various influence(s) that single TFs exert, including aspects of

organogenesis, progenitor cell proliferation, retinal lamination, and even the fate specification of single cell types.

Since these discoveries, it has become apparent that not only one, but a regimen of factors, expressed as a transcriptome, are required to achieve the high degree of fidelity seen during the production of different neuron subtypes in the post-mitotic retina (see Hatakeyama and Kageyama, 2004). The combinatorial expression of both bHLH and HD transcription factors is crucial for the initiation of the genesis of all classes of retinal cells, as summarized in Figure 1-4. Much like the cross-repressive actions of TFs identified and characterized in the development of ventral spinal neurons (Edlund and Jessell, 1999; reviewed by Jessell, 2000), interaction between transcriptional activators and repressors result in a number of possible TF combinations. In addition, although it is known that extrinsic factors can influence TF expression, as is seen in delta/notch mediated expression of Hes1, the precise mechanisms involved in this phenomenon are not completely understood.

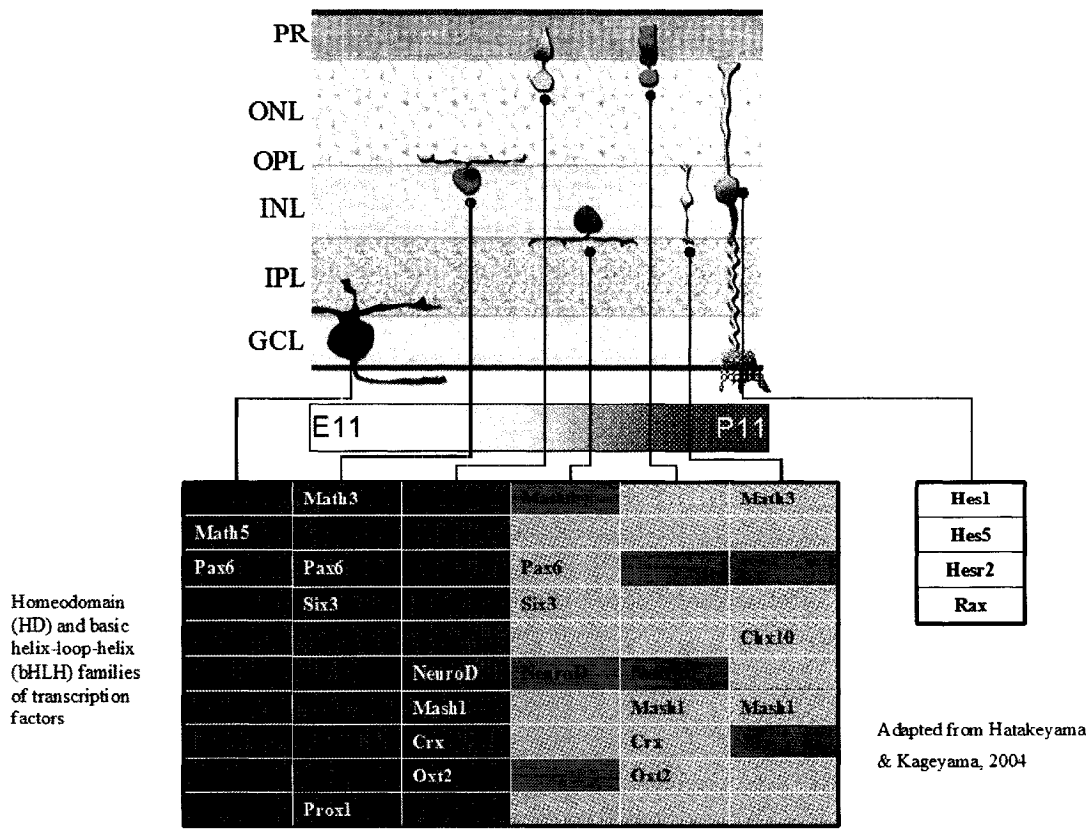


Figure 1-4. Transcription factors and the diversity of retinal cell production. The development of each retinal neuron subtype is dependent on the intrinsic expression of particular TFs within individual RPCs (shown here for the mouse). The earliest cell type produced in the retina, the RGC, requires that Math5 and Pax6 are co-expressed in its precursor cell. The production of each retinal cell subtypes requires its own particular transcriptome. Many common TFs are involved in the production of different retinal neurons, whereas the Müller glial cell requires a distinctly different TF complement, closely resembling that which is expressed in active RPCs. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; PR, photoreceptors.

The Role(s) of Diffusible Factors in Fate Determination and Cell Cycle Dynamics During Retinal Development

Throughout the three week retinogenic window in rodents, a dynamic shift in RPC competence is correlated with changing intrinsic gene expression therein, and with alterations in molecular cues derived from the extracellular milieu. The neuroblastic bilayer of epithelia present in each optic vesicle at E10.5 contains precursors that will give rise to either retina (outer layer) or RPE (inner layer). Although the ultimate fate of progeny derived from these two populations of progenitors will be distinct (retina vs. RPE), both are endowed with the competence to generate a fully formed retina (discussed in Sakaguchi et al., 1997). Our understanding of why these two equipotential populations differentiate into distinctly different structures was elaborated by carefully dissecting away adjacent ocular structures, and examining the resulting retinal phenotype (reviewed by original author in Holtfreter, 1951). It has become evident that signaling from adjacent paraocular mesenchyme and surface ectoderm via diffusible factors contribute to the polarity of fate exhibited by RPE and RPCs (Fuhrmann et al., 2000). In addition, the removal of both epithelia and their re-implantation 180 degrees relative to the original position results in the reassignment of the fate of RPE primordia, to that of retinal tissue (reviewed in Chow and Lang, 2001). It was later identified that signaling via FGF-2 initiated in RPCs, and derived from the surface ectoderm, stimulates retinogenesis by RPCs but *not* RPE progenitors. Specifically, removal of the surface ectoderm inhibits retinogenesis, an effect that can be restored with the application of FGF-2. The blockade of FGF-2-signaling by the application of FGF-2-specific antibodies recapitulated the results seen in surface ectoderm removal, suggesting either direct FGF-2 signaling, or

paracrine induction of FGF-2 signaling within the retina, was derived from ectodermal cues.

The identification of extrinsic factors involved in directing retinal fate specification continues. Beyond that of specifying gross retinal fate within the oculus, extrinsic factors have been shown to contribute to the specific production of various retinal cell types. This concept is underscored by the demonstration that newly generated RGCs provide diffusible feedback inhibition that regulates their further production (Gonzalez-Hoyuela et al., 2001). This study showed that accumulating numbers of RGCs results in a decrease in RGC production, and that this effect is mediated by the release of nerve growth factor (NGF) by RGCs. A similar effect was seen *in vitro*, wherein RPCs were re-aggregated with enriched populations of individual retinal neuron subtypes (Belliveau and Cepko, 1999). This work investigated the influence of the extracellular milieu on the fate specification of individual cell types. It was discovered that a non-amacrine derived, extrinsic factor was capable of influencing the type of cell generated by RPCs (Belliveau and Cepko, 1999). Results from these studies imply that extracellular factors can, to some extent, stimulate a switch in competence states by RPCs from one that involves the production of one cell type to that of another. However, as mentioned above, extracellular cues are not sufficient to alter the production of all cell types by RPCs and, evidently, act only to partially modulate the delicate repertoire of retinal cell production.

A number of diffusible factors, mainly those identified as growth factors, have since been shown to exert significant influences on proliferation and competence of RPCs (reviewed by Livesey and Cepko, 2001a). Those factors include epidermal growth factor

(EGF; Lillien, 1995), bFGF (Pittack et al., 1997), acidic fibroblast growth factor (FGF-2; Guillemot and Cepko, 1992), Delta (Austin et al., 1995), CNTF (Ezzeddine et al., 1997), taurine (Altshuler et al., 1993), sonic hedgehog (shh; Zhang and Yang, 2001b), LIF (Neophytou et al., 1997), NGF (Gonzalez-Hoyuela et al., 2001) and retinoic acid (RA; Kelley et al., 1994).

Stem/Progenitor Cells of the CNS

The Origin of the CNS Stemness Concept

There are few scientific and/or medical concepts evident in today's global psyche which elicit the vigorous public response that "stem cell" research has. Its widespread therapeutic ramifications, if proven to be effective, stimulate a range of opinions regarding public support of the science. Those opinions are often shaped by moral, ethical, biological and religious considerations. The various sources of stem cells raise considerable debate between purveyors of therapeutic science and eager patients in queue for revolutionary treatments, and theologically and philosophically based thinkers. The result of these tensions has been the search for effective stem cell treatments derived from less controversial sources such as the adult donor, or individual patients themselves as autologous donors.

Stem cells began to move from being a largely botanical concept, into the realm of animals with their discovery in differentiated invertebrate tissues (for review, see Andrews, 2002). A number of stem cell categories have since been defined based on criteria such as tissue origin, phylogenetic and developmental stage of the source animal, their potential to generate different lineages of tissue types, and whether or not they are part of the animal germ line (reviewed by Bongso and Richards, 2004). With respect to

the CNS, it was thought that following the termination of development, the CNS was not only devoid of neurogenic activity, but also lacked any neurogenic potential. These conclusions were derived from observations that the post-developmental brain, spinal cord and retina were not only postmitotic, but lacked any evidence of cell replacement following pathogenic insults. Seminal studies using novel lineage tracing techniques used to identify proliferating cells and their progeny, however, identified two proliferating regions in the brain that persist into adulthood (Altman and Das, 1965b; Altman and Das, 1965a; Altman and Das, 1966). Phenotyping of progeny derived from this cell proliferation established that newly generated cells derived from these areas migrate as neuroblasts (cells destined to become neurons) into the olfactory bulb and hippocampus, and differentiate into cells that resemble postmitotic interneurons. This revolutionary series of observations changed perspectives regarding what regenerative potential may lay dormant within the brain, spinal cord and retina. Subsequent to this, extensive research has elucidated the phenotypic and functional characteristics of the cells responsible for neuron production in these areas.

Current Concepts and Frameworks Used in Determining Stemness

By the mid 1990s, precise dissection of neural tissue combined with *in vitro* culture techniques had provided a well established assay for the identification and characterization of "stem cell" properties. Specifically, tissue was procured, dissociated and seeded into culture plates via either a monolayer assay (Palmer et al., 1995) or a colony-forming neurosphere assay (Reynolds and Weiss, 1992). It was apparent from these studies that harvested cells had a robust proliferative capacity, as well as the ability to generate progeny in all three neuroectodermal lineages (i.e. neurons, astrocytes and

oligodendrocytes). This ability to generate multiple cell types (multipotentiality) is reminiscent of that which is seen in populations of neural precursors active during neural development. More impressive was the discovery that these "stem" like cells could clonally divide to generate daughter cells that retain that very same multipotentcy, a process coined as "self-renewal". Differences in these functional attributes became apparent, however, upon comparison of cells dissected from the ventral forebrain with those derived from the hippocampus. Cells dissected from the hippocampus displayed a limited capacity for both self-renewal, and multilineage cell production (Seaberg and van der Kooy D., 2002). These observations revealed an emerging framework for describing not only the various cellular isotypes seen in proliferating zones of the adult CNS, but also the varying degree of "potential" of cells that display stem-like characteristics. This framework was established using criteria based on a cell's ability to self-renew, as well as "restrictedness" with respect to its ability to generate multiple cell types via cell division. Thus, new terminology aimed at distinguishing between these different cell potencies, and a critical reevaluation of the semantics used in their description, have become prominent issues in stem cell science.

To date, there are a number of proposed frameworks which attempt to clarify the concept of "stemness" in both tissue-specific, and temporal-developmental contexts. One generally accepted framework of stemness is reviewed by Seaberg and colleagues (Seaberg and van der Kooy, 2003), and will be ascribed to throughout this thesis. Briefly, a true multipotent CNS stem cell exhibits the capacity to clonally divide to generate copies of itself which retain the same multipotency (self-renewal). In addition, it must be able to divide to generate cells from all three neuroectodermal lineages (multipotentiality

mentioned above). Clearly, this list of requirements is particularly demanding, and correlates with the rarity of these cells within the CNS. More prevalent within the CNS are cells with varying and limited degrees of potential relative to the capacities of a true CNS stem cell. CNS "progenitor cells", for example, exhibit a robust capacity to generate multiple cell lineages, but retain only a limited ability to self-renew. Even more restricted "precursor" cells can divide to generate viable progeny, but they are restricted to generating only one specific cell type and are, thus, not awarded the prestigious title of either stem or progenitor. For the purposes of general discussion, and out of respect for the semantics involved in defining these distinct categories of cells, I will refer to all cells that can divide to generate viable progeny (of neurons or glia), and that may or may not self-renew, as "neural precursor cells" (NPCs).

In addition to self renewal and potential, specific molecular characteristics have been shown to be consistent amongst populations of NPC. These characteristics include: active janus kinase signaling; responsiveness to growth factors; re-entry into the cell cycle; enhanced DNA repair mechanisms and resistance to DNA damage; and DNA methylase and histone deacetylase sensitivity (for review, see Bongso and Richards, 2004). The desire to identify neural precursors both *in vivo* and *in vitro* has fueled the use of extensive screening assays in an effort to elucidate single cellular phenotypes exhibited by these cells. Unfortunately, no single phenotype has been identified that unequivocally identifies NPCs; however, a select panel of phenotypes is consistently present and can be used in combination to help identify candidate cells. As presented in this dissertation, I rely largely on antibodies that identify these phenotypes, and correlate their expression with progenitor-like activity of cells within the retina.

Adult Neural Stem/Progenitor Cells

Following the identification of proliferating zones in the adult mammalian brain (Altman and Das, 1965b; Altman and Das, 1965a), considerable effort has been focused on identifying sources of cell replacement within the adult CNS. It was apparent in lower-vertebrates that resident precursors involved in metamorphosis from young to adult are also capable of generating and modifying a variety of adult tissue types and organs, including the CNS (for review, see Endo et al., 2007). In addition, these cells respond to injury in such a way that functionally significant regeneration is possible. In contrast, the mammalian CNS displays no significant regenerative response to injury, and CNS pathologies (both chronic and acute) inevitably result in profound and irreversible neuronal dysfunction and/or death. Since the race to identify substrates for adult neural cell replacement began, cells that display characteristic features of neural precursors have been identified in a number of brain regions (Sohur et al., 2006). These cells range in potential from *bonafide* neural stem cells to proliferating neural precursors. It is clear, however, that many of the cardinal characteristics that we demand when scrutinizing their authenticity in terms of stemness, are only inducible *in vitro*. Recent studies *in vivo* have reported the ability to activate neurogenic regions, specifically that of the hippocampus and cortex, and generate neuronal progeny that integrate into pre-existing circuits (Ramirez-Amaya et al., 2006; Toni et al., 2007; Magavi et al., 2005).

The Müller Glial Cell

Functions of Müller Glia

The development of the CNS depends greatly on the coordinated efforts of neural progenitors, as well as support cells retaining cardinal features of astrocytes. In the

development of the cerebral cortex, these specialized glia, termed radial glia, are characterized by distinct morphological, chemical and functional properties. The induction of the radial glial phenotype by cells of the neural tube correlates with the onset of neuron production by adjacent cells (Feng et al., 1994), outlining the importance of coordinated developmental timing between these two cell populations. Our understanding of radial glial function during development was benefited, in large part, by seminal experiments spearheaded by Rakic and colleagues. These experiments demonstrated that the function of these cells is almost exclusively restricted to providing a substrate for the radial migration of newly generated neuroblasts (reviewed by original author in Rakic, 2003). The expanse of the radially oriented processes span the ventricular and pial zones, which permits access of migrating neuroblasts to all layers of the developing cortex. This close association between migrating neurons and radial glia also provides a convenient microdomain for intercellular signaling. This radial glial/neuroblastic crosstalk has been identified, and is now implicated in the modulation of fate specification in maturing, migrating forebrain neurons (Toresson et al., 1999). One example of this is seen in the development of medial versus lateral ganglionic eminence neurons. Radial glia of the medial component produce retinoic acid, unlike their lateral component counterparts. This retinoic acid signaling results in divergent fate specification of medial vs. lateral ganglionic eminence neurons destined to become projection neurons of the striatum (Toresson et al., 1999). Recent results have identified a far more intimate and active role for radial glia in the process of forebrain neurogenesis. Work pioneered by Götz and colleagues has shown that radial glia are actively neurogenic in rodents, and that their capacity to generate neurons is governed by similar transcriptional mechanisms to those

identified in neuronal progenitors during very early phases of neurulation (reviewed by original author in Gotz and Barde, 2005).

The retina contains its own class of radial glial cell termed the Müller cell. Müller glia are the exclusive glial support cell of the retina (for a comprehensive review, see Bringmann et al., 2006). Müller glia are classified as "radial" due in part to their elongated, serially arranged and parallel morphology that collectively span most of the retina, from the inner to outer limiting membranes (see Figure 1-1). Features of Müller glial cells that are akin to their counterparts in the developing forebrain include cytoplasmic glycogen granules, as well as the expression of glial fibrillary acidic protein (GFAP), brain lipid-binding protein (BLBP), astrocyte-specific glutamate transporter (GLAST) and, in some cases, nestin. The functions of Müller glia in the adult retina include providing substrates (glucose) for oxidative metabolism (Poitry-Yamate et al., 1995), regulating blood flow as well as contributing to, and maintaining the blood-retinal barrier (Tout et al., 1993), providing precursors for the synthesis of neurotransmitters by neurons and the rapid uptake and recycling of neurotransmitters such as glutamate (thus facilitating retinal processing) (Matsui et al., 1999), maintaining extracellular concentrations of ions such as potassium via prodigious numbers of K^+ channels and regulating H_2O gradients (Newman and Reichenbach, 1996), releasing neurotrophins (Harada et al., 2002), and modulating neuronal excitability via the cellular release of glutamate (Newman and Zahs, 1998).

Müller Cell Response to Injury

The functions of Müller glia are extended in response to pathogenic retinal conditions. A reactive gliotic response, coupled with a particularly high resistance to cell

death, extend the active role of Müller glia to include attempting to restore retinal homeostasis and repair following damage (Bringmann et al., 2006). Müller glia ensheath all retinal neurons, which provides a convenient microdomain for signaling and supportive functions during pathological events. The array of functions following injury range from mediating the inflammatory response, to phagocytosing fragments of lysed retinal neurons. Müller glia can mediate the recruitment of microglia , receive neurotrophin-releasing signals from microglia, and release pro-inflammatory cytokines, highlighting their intimate role in the inflammatory response (Harada et al., 2002). In some conditions, the release of neurotrophins and antioxidants is evident, presumably in an attempt to protect retinal neurons from further damage (Harada et al., 2002). The buffering of neurotransmitter release also serves to diminish the excitotoxic effects of events such as glutamate release (reviewed in, Bringmann et al., 2006). Hallmark features of reactive gliosis are also exhibited by these cells, including up-regulation of GFAP, somatic swelling, and in some instances, re-entry into the cell cycle.

In addition to a supportive role in retinal homeostasis, Müller glia can also contribute to the damage cascade, an effect that exacerbates the pathological condition of the retina. For example, Müller cells help to maintain the integrity of the blood retinal barrier (Tout et al., 1993). In pathological conditions involving vasculature, such as diabetic retinopathy, secretion of hormones such as vascular endothelial growth factor compromise the selective permeability of these structures (Pierce et al., 1995). Specifically, vascular endothelial growth factor overproduction contributes to the leakiness of retinal vasculature, an observation that clearly exemplifies the sometimes antagonistic, or detrimental influence that Müller cells can exert. An extreme phenotype

elicited by Müller glial reactive gliosis is the formation of retinal dysplasia. Exogenously induced and inherited retinal disease which induces widespread Müller cell gliosis results in their re-entry into the cell cycle, and dramatic changes in their cellular ultrastructure (Dyer and Cepko, 2000). The result of these changes is a disruption in the outer limiting membrane, which is normally formed by the apical processes of Müller cells. In summary, although Müller glia do perform as efficient regulators of retinal homeostasis, their contribution to the pathogenic response to injury by the retina can have profound effects on many aspects of retinal integrity.

Müller Glia as Neural Precursors

During development, Müller glia are the last cell type produced (Young, 1985). Their morphology is reminiscent of that exhibited by individual RPCs that make up the pseudostratified retinal neuroepithelium. They are positioned within a developmental micro-unit, which itself is comprised of a column of neuronal progeny derived from a single RPC and a centrally located Müller cell (Turner and Cepko, 1987), similar to that which is seen with regard to Bergmann glia in the cortex of the cerebellum (Reichenbach et al., 1995). By P10.5 in mouse, remaining RPCs have exited the cell cycle, and have subsequently down-regulated phenotypes typically associated with RPC function. It was proposed that during this period of differentiation, remaining RPCs up-regulate phenotypes akin to those expressed by adult Müller glial cells. This would also imply that Müller glia are the direct lineage decedents of RPCs and thus, may retain some progenitor-like potential. Proof of concept experiments in mice using retroviral lineage tracing methods examined this possibility (Turner and Cepko, 1987). This technique allows for the infection of a single RPC with the β-galactose gene, and the identification

of resulting progeny generated by that RPC in the form of X-Gal-positive clones (Cepko et al., 1998). At P7, a time at which the RPC population initiates its last round of cell division, rod and Müller glial clones were generated. This observation suggested that the final division of RPCs is asymmetrical, and that the non-neuronal progeny (presumably the RPC) adopts the Müller glial phenotype. These results prompted examination of whether Müller glia retain any functional RPC-like characteristics, including that of neurogenic competence. As will be discussed in detail below, RPC behavior and neurogenic competence by Müller glia has been clearly identified in a number of vertebrate species, including those belonging to teleost (for review by original author, see Raymond and Hitchcock, 1997), avian (Fischer and Reh, 2001) and mammalian (Ooto et al., 2004) classes. This capacity is quiescent, however, and requires exogenous activation via exposure to either pathogenic insults (Ooto et al., 2004), exogenously applied growth factors (Fischer et al., 2002b) or alterations in intrinsic gene expression (Moshiri and Reh, 2004; Ooto et al., 2004).

Neurogenic Müller Glia in the Chick Retina

The field of adult retinogenesis has been revolutionized by the coordinated work of Andy Fischer and Tom Reh. Their use of the highly accessible chick retina model has provided valuable insight into adult retinal precursors (Fischer and Reh, 2001; Fischer et al., 2002b; Fischer and Reh, 2003a; Fischer and Reh, 2003b; Fischer et al., 2004; Fischer and Omar, 2005). The first demonstration of Müller glial-derived neurogenesis was in response to the selective damage of specific subsets of retinal neurons induced via the exogenous application of excitotoxic compounds (Fischer and Reh, 2001). The cell-specific and profound pathology induced by NMDA and colchicine treatment resulted in

a wave of proliferation within the INL, a process that was predictable, time-dependent, and varied as a function of the developmental stage (for review, see Fischer and Reh, 2003b). When assayed, the proliferating cell type was identified as being predominantly Müller glial; however, a sub-population of remaining progeny were not. Further evaluation using pulse/chase lineage analysis determined that some newly generated cells (< 4%) eventually express immunohistochemical markers associated with maturing neurons (Hu C/D, calretinin or cellular retinoic acid-binding protein), demanding the conclusion that proliferating Müller glia were, in fact, neurogenic. In addition, dividing nuclei of Müller glia underwent a process of interkinetic nuclear migration, which is characterized by the intracellular movement of nuclei across the nuclear and plexiform layers of the retina. This behavior is a functional phenotype seen in proliferating CNS progenitors (reviewed in Tramontin et al., 2003), including RPCs, during development. These observations prompted a robust assessment of the responsiveness of Müller glia to a variety of stimuli previously shown to induce neurogenesis in other CNS neural precursors.

As mentioned above, RPCs express a very specific regimen of genes that can be used in their identification during development. Examination of retinas containing activated, neurogenic Müller glia determined that these cells co-express Pax6 and Chx10, transcription factors that are normally co-expressed in RPCs during development (Fischer and Reh, 2001). When treated with NMDA, the up-regulation of Pax6 and Chx10 within dividing Müller glia were co-incident with the expression of neurofilament, exemplifying the high degree of phenotypic plasticity of these cells. It was discovered that a population of Chx10/Pax6 labeled cells, not identifiable as Müller glia or mature neurons, persisted

following treatment. These results indicate that, despite retaining RPC-like characteristics, neurogenic re-population following Müller glial activation is challenged by salient cues within the adult retinal environment that discourage the efficient differentiation of progeny into neurons.

Mammalian Müller Glial Neurogenesis: An Emerging Field

The race to identify sources of therapeutic cell replacement within the adult retina began with lower vertebrates, and has steadily climbed up the phylogenetic tree. Ultimately, the goal of cell replacement science is to generate therapeutically relevant treatments in the context of human retinopathies and CNS disease. Evidently, recent advances in this field using a variety of *in vivo* and *in vitro* methods have demonstrated limited neurogenic potential within mammalian (rodent) Müller glia. There exists a consistent phylogenetic trend of Müller cell neurogenic competence, whereby "lower" vertebrates exhibit a much higher degree of neurogenic activity relative to "higher" vertebrates (reviewed by Moshiri et al., 2004). In accordance with this trend, mammalian Müller glia have exhibited a particularly low-level response to retinal injury when compared to their lower vertebrate counterparts. This frustrating inability to re-capitulate earlier results attained in both the teleost and avian retinas has lead to the development of novel approaches. These approaches include the use of an expanding regimen of epigenetic factors that are effective in activating other populations of NPCs, and the manipulation of intrinsic gene expression. Both of these approaches attempt to kick-start Müller glia into up-regulating the necessary machinery required to exit the quiescent state, and become neurogenic.

To date, only two significant publications have described any effective neurogenic response by adult Müller glia in "higher" vertebrates. Ooto and colleagues used excitotoxic activation protocols previously described in chick to induce Müller cell re-entry into the cell cycle (Ooto et al., 2004). They then examined the influence of retinoic acid, and the misexpression of bHLH genes on the fate of newly generated progeny. Their findings identified that adult Müller glia can be neurogenic when gene expression is altered to favor neuron production, as seen following forced expression of pro-neuronal genes. A second group lead by Iqbal Ahmad recently analyzed the stem cell potential of cultured mammalian Müller glia using the well established neurosphere assay (Das et al., 2006). This group was the first to demonstrate self renewal and multipotentcy by Müller glia. Interestingly, the stem cell like characteristics of these cells involved the up-regulation of notch and wnt signaling pathways, and could be enriched via excitotoxic injury to the retina. As will be discussed in this thesis, enrichment of progenitor activity within Müller glia is evident following various forms of injury, and links mechanisms of glial reactivity with progenitor activity in the CNS.

Manipulation of Stem/Progenitor Cell Function

Directing the Fate of CNS Progenitors In vitro and In vivo

Generating efficacious treatment strategies for cell replacement in the CNS will likely require clinicians to be able to efficiently and accurately manipulate the behavior of source NPCs. In my opinion, NPC science has accomplished two major advances to date: (1) the discovery of candidate NPC populations and assessment of their potential to generate various neural progeny; and (2) manipulation of their proliferative activity (that is, being able to turn them "on" and "off") and directing the fate of progeny generated

from that proliferation. One can also argue that impressive strides have been made towards a third achievement, one that falls outside of the focus of this thesis: encouraging these cells to migrate to desired positions and integrate into existing circuits.

As mentioned above, control of stem/progenitor cells can be achieved via manipulation of either of two dominant signaling domains: (1) intrinsic expression of appropriate pro-neural genes using viral misexpression constructs; and (2) conditional overexpression of candidate genes in cells of interest using Cre-loxP transgenic mice. Although these techniques are incredibly powerful tools that are useful in elucidating the function(s) of particular genes of interest, and in particular cells of interest, their role in clinical cell replacement may be limited. Complications with viral constructs vary depending on the host from which they were derived, and include limitations such as the requirement of some vectors to target cells engaged in the cell cycle, immunoreactivity toward infected cells and cytotoxicity (for review, see Mancheno-Corvo and Martin-Duque, 2006).

The pharmacological manipulation of cells *in vivo* has been a dominant modality of therapeutic medicine for centuries. The pharmacological sciences have focused their energy on generating compounds that display amplified potencies, increased targeting accuracy towards cells of interest, and prolonged lifespan once introduced to the organism. The wide variety of analogues available to scientists today has fueled their use in manipulating biological systems, and the area of NPC science is certainly no exception. The use of growth factors, and growth factor analogues has been paramount in the discovery of basic strategies for the manipulation of NPCs. A long list of mitogenic and morphogenic compounds shown to influence various aspects of cell cycle dynamics,

fate determination and survival has been established, including: bFGF, CNTF, transforming growth factor α (TGFα), shh, RA, LIF, brain-derived neurotrophic factor (BDNF), erythropoietin (EPO) and EGF to name a few (for review, see Hagg, 2005). BDNF, a neurotrophin used in experiments presented in this thesis, signals through the receptor tyrosine kinase (Trk) class of receptors, and has been traditionally studied as a neurotrophic factor (reviewed in, Nagappan and Lu, 2005). BDNF binds to one of two Trk receptors, the high affinity TrkB receptor, and the low affinity P75 growth receptor. Its involvement in neural development, includes that of the retina where it plays a central role in RGC survival during critical periods of target innervation (Ma et al., 1998). During the mid 1990s, there was a significant push by NPC scientists to identify candidate factors that could be used to increase the yield of neurons produced by adult NPCs. This was to be achieved by encouraging both neuronal differentiation and survival. The survival-promoting effect of BDNF on NPCs was tested *in vitro*, with interesting results. It was observed that rather than exerting a trophic effect *per se*, BDNF was able to promote neuronal differentiation (Shetty and Turner, 1998), subsequently leading to a series of studies characterizing this property both *in vitro* (Ahmed et al., 1995; Arsenijevic and Weiss, 1998) and *in vivo* (Zigova et al., 1998; Pencea et al., 2001; Scharfman et al., 2005).

Epidermal Growth Factor (EGF)

Soluble factors mediate innumerable aspects of cell function during development, including that of proliferation, survival, and differentiation. Their discovery and functional characterization classically involved their purification, and subsequent re-introduction into the animal at high concentrations. One of the first and most widely

studied of these is EGF, which was purified and characterized by Cohen and colleagues in 1962 (COHEN, 1962). His seminal studies discovered that urogastrone, later termed EGF, was able to influence the development of dentition, as well as keritanization of skin (COHEN, 1962). Its influence on the proliferative dynamics of skin cells was described a year later (COHEN and ELLIOTT, 1963). Over four decades since those discoveries, EGF has been implicated in mechanisms involved in human proliferative and non-proliferative pathologies ranging from psoriasis (Ben Bassat and Levitzki, 2000) to cancer (Araujo et al., 2007).

EGF, and its receptor EGFR, belong to the family of receptor tyrosine kinases. The EGF family of ligands include TGFα, amphiregulin, heparin-binding EGF-like growth factor, betacellulin and heregulin, only a small subset of which bind to the EGFR (for review, see Wong and Guillaud, 2004). EGF signaling can involve the activation of PLCγ, Jak-STAT, raf-MAPK and/or PI3-kinase intracellular pathways. Roles for EGF in the development and functioning of the CNS were evident when McKanna and Cohen discovered EGFR ligands in the floor plate of the developing rat (McKanna and COHEN, 1989). Since this discovery, EGF signaling has been shown to be critical in many aspects of neural morphogenesis (Wong and Guillaud, 2004).

In the realm of adult NPC science, EGF became widely popular due to its role in the discovery of adult neural stem cells by Reynolds and Weiss in the early 1990s (Reynolds and Weiss, 1992). EGF and its receptor are widely expressed in the developing nervous system (Fallon et al., 1984), and deficits in EGFR induced in transgenic mice results in lethality, ranging from embryonic to P20 (for review, see Wong, 2003). It is interesting to note that mutants exhibit deficits in neuroepithelial proliferation, which

result in reductions in the volume of a variety of brain structures (Threadgill et al., 1995). By the early 1990s, the activity of EGF with respect to early development of neural tissue had been described (Morrison et al., 1987; Simpson et al., 1982). Together, these data predicted that EGF could be used to activate NPCs, if they did in fact exist in the adult brain, and eventually lead to the discovery of adult neurogenic substrates by Reynolds and Weiss (Reynolds and Weiss, 1992).

Similar predictive data are evident in the retina with respect to NPC activation and EGF. Although EGF is not synthesized in the retina proper, its expression is evident in many paraocular tissues (Anchan et al., 1991), and its receptor is highly expressed on RPCs (Lillien, 1995). Laura Lillien was the first to assay for the role of EGFR in the developing retina (Lillien, 1995; Lillien and Wancio, 1998). These early reports described changes in the cell fate of newly generated progeny, and not proliferation *per se,* following the insertion of extra copies of the EGFR into active RPCs. In the adult retina, EGF has been shown to influence the proliferation of Müller glia during late stages of retinal development (Close et al., 2006), and in culture (Scherer and Schnitzer, 1994). Investigation into the potential influence of EGF on adult mammalian Müller glia and their possible progenitor-like response, however, had not been described. As will be discussed in this dissertation, I describe RPC-like behavior by these cells in response to exogenously applied EGF (Chapter 3), suggesting that Müller cell de-differentiation may involve a similar mitogenic response to that seen in populations of adult brain NPCs.

Erythropoietin (EPO)

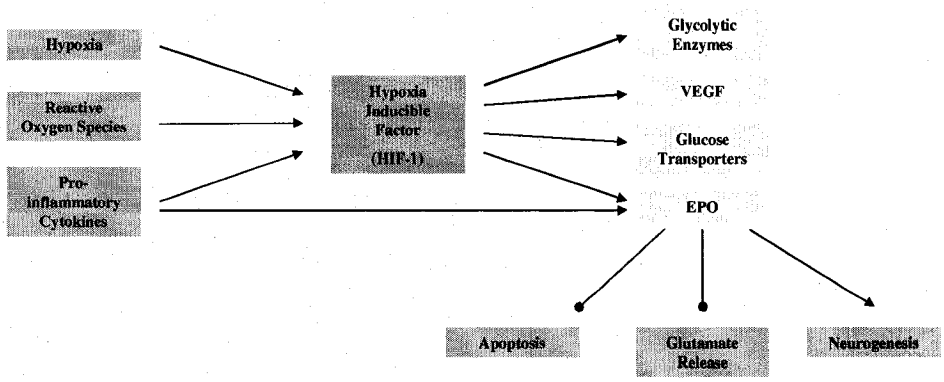
In 1998, the global public was inundated with media coverage on the "Tour de Shame", a scandalous eruption which implicated a large portion of the world's top

cyclists as users of performance enhancing drugs during the Tour de France. "EPO" became a common term when discussing cyclists, and the basic understanding of its performance enhancing effects became common knowledge amongst young athletes. A number of high-profile French and non-French athlete deaths related to long-term EPO abuse causing cardiac failure prompted the World Anti-Doping Agency to institute an aggressive anti-EPO campaign (much to the dismay of some cyclists).

EPO is a pleiotropic glycoprotien hormone synthesized primarily in the kidney, and is classically identified as a factor involved in erythrocyte production, maturation and survival (for review see Yoshimura and Arai, 1996). Since its purification by Miyake and colleagues in 1977, this ~30 kDa, type-1 cytokine has been cloned into recombinant form, and modified into a variety of synthetic analogues that are currently used clinically in the treatment of anemia. Widespread treatment of anemic patients has proven to be extremely successful, and has launched human recombinant EPO and its synthetic analogues into the top ranks of pharmaceutical sales (Jelkmann, 2004). Its potent and long-lasting ability to boost the hematocrit, and therefore the oxygen carrying capacity of blood, is the primary functional benefit sought after by aerobic athletes such as those in the "Tour".

The regulation of EPO production occurs predominantly within the cortex of the kidneys, and involves oxygen-sensitive mechanisms mediated by a positive EPO transcriptional regulator, HIF-1α (Figure 1-5; for review, see Jelkmann, 2004). Under normoxic conditions, HIF-1α, a member of the bHLH family of transcription factors, is continually tagged for proteolytic degradation which inhibits its translocation to the nucleus. During hypoxic tissue conditions, however, HIF-1α is permitted to enter the

nucleus and bind to hypoxia response elements (HRE) located upstream of the EPO gene, thus activating EPO gene expression. It has become increasingly clear, however, that EPO production can be stimulated by factors that are separate from classical hypoxia-related mechanisms.



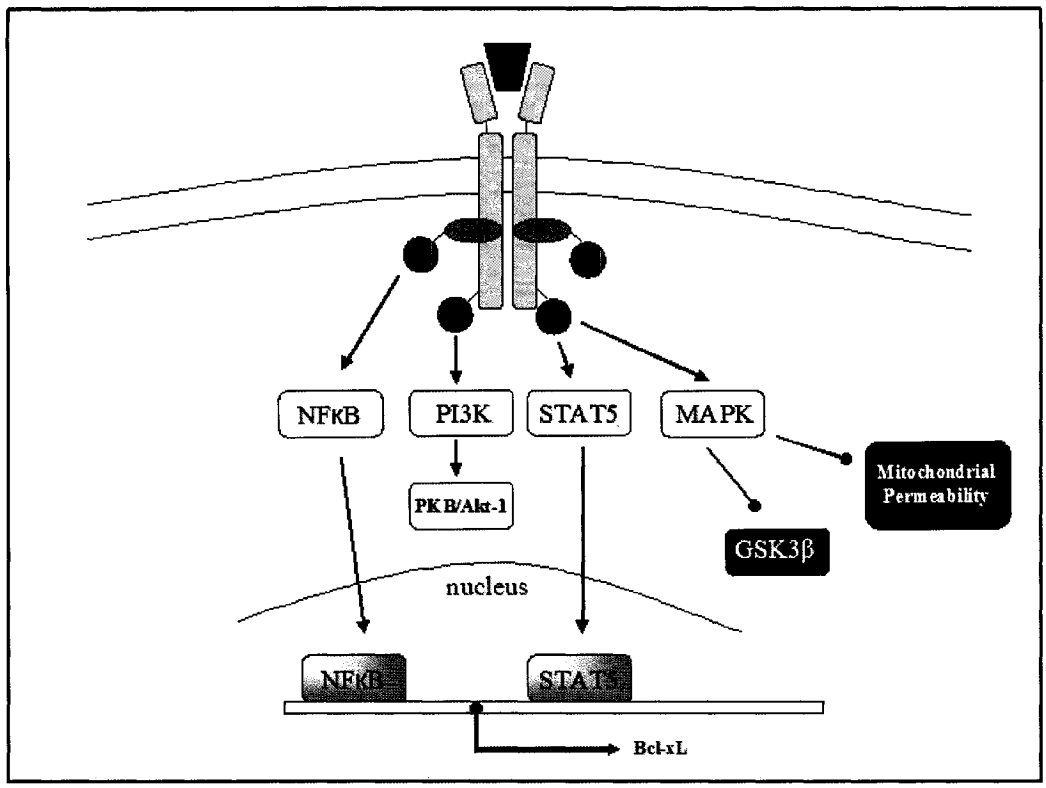
Adapted and modified from Brines & Cerami, 2005

Figure 1-5. Regulation of EPO production. The hypoxia inducible factors (HIF) are regulatory TFs that bind to hypoxia response elements (HRE) upstream of the EPO gene. HIF-1 activation of HREs can be induced following low-oxygen, and pathologically-induced production of reactive oxygen species and pro-inflammatory cytokines. The HIF-1 transcription factor also activates a variety of responses associated with the tissue response to damage, including those involved in energy metabolism and growth factors. One such growth factor, EPO, when expressed can modulate cell survival by inhibiting apoptosis and in addition, neurotransmitter release. When exogenously applied, EPO can influence the fate of newly generated cells in brain.

A number of factors released in tandem with tissue damage or hypoxia brought upon by ischemia, such as reactive oxygen species and pro-inflammatory cytokines, have been shown to increase both the activation of HIF-1α and, in some instances, the tissue production of EPO (Figure 1-5; reviewed by Brines and Cerami, 2005).

EPO Signaling

Signaling by EPO (Figure 1-6) is initiated following binding to its receptor EPO-R, which is a member of the class-1 cytokine family of receptor tyrosine kinases (reviewed in Jelkmann, 2004). Binding of the EPO ligand results in the homodimerization of two EPO-Rs, and a conformational change of the EPO-R homodimer that increases the proximity of their intracellular domains. Each intracellular portion harbors a janus kinase 2 (Jak2) tyrosine kinase protein involved in intracellular signal transduction. Transphosphorylation of Jak2 results in the phosphorylation of tyrosine residues present on the intracellular portion of the receptor homodimer complex, the consequence of which is activation of one of 4 dominant EPO-signaling pathways (NFκB, MAPK, PI3K and STAT5).



Adapted and modified from:
Bartesaghi et al., 2005 and Brines & Cerami, 2005.

Figure 1-6. EPO signaling. The binding of EPO to its receptor, EPO-R, results in activation of 4-possible signaling pathways which include: NFκB, PI3 kinase, STAT5 and MAPK. Downstream effects of EPO signaling range from activation of anti-apoptotic Bcl genes, to modulation of mitochondrial permeability. Although the signaling mechanism is only partially understood, activation of the NFκB pathway following exogenous EPO treatment in rodent brain NPCs increases the production of neurons, and is thought to involve the downstream up-regulation of the neurogenic transcription factor, MASH-1.

EPO in the CNS

EPO was first discovered in the CNS in the early 1990s (Konishi et al., 1993;Tan et al., 1992) and, consistent with its mechanism of expression in the kidneys, EPO mRNA within CNS cells was up-regulated following a reduction in extracellular oxygen levels (Masuda et al., 1994). Working concurrently, two separate groups characterized the cellular production of EPO in astrocytes (Masuda et al., 1994) and its ability to exert neuroprotective effects on cholinergic projection neurons in brain (Konishi et al., 1993), indicating a biological role for EPO in the CNS. EPO and EPO-R localization in the human fetus has been studied, both being present throughout the developing CNS (Juul et al., 1998). In terms of the retina, EPO-Rs are found on photoreceptors, cells of the INL (Grimm et al., 2002), and RGCs (Weishaupt et al., 2004). Their presence on photoreceptors and RGCs has been show to be key in the neuroprotective effects of exogenously applied EPO following experimental injury (for review, see Coleman and Brines, 2004).

Beyond the realm of neuroprotection, EPO has also been used to promote the genesis of neurons from active NPC populations in the adult brain (Shingo et al., 2001). Shingo and colleagues demonstrated that chronic infusion of EPO into the lateral ventricles induced the differentiation of non-dividing, neural stem cells into migrating neuroblasts. Analysis of germinal zone progenitors in E14 mice, as well as SVZ NPCs in adult, showed that EPO-Rs are present in these areas, and co-localize with EGF receptors that are known to be highly involved in NPC proliferation. Research from the Weiss lab has been extended into a paradigm that includes EPO as a treatment strategy for stroke. Specifically, exogenous EGF/EPO treatment stimulates SVZ NPCs to repopulate cortical

areas that have been damaged via a stroke model. This repopulation results in functional behavioral recovery in rodents (personal communication), although the etiology of that recovery is still of some debate.

As will be addressed later in this dissertation, we describe the influence of exogenously applied EPO on the proliferation and phenotype of adult mammalian Müller glia. In addition, we employ a similar activation protocol to that which is used by the Weiss lab, wherein EGF/EPO are introduced in combination to the retinal environment in an effort to activate complementary progenitor mechanisms in the eye.

Goals of the Thesis

The emerging field of adult neural precursor biology has spawned an epidemic of research centered on CNS-focused, cell replacement science. It is clear that the adult mammalian retina contains *bonafide* retinogenic potential. Harnessing this potential, and characterizing the response of endogenous neural precursors to both pathological conditions and to exogenous activation, will prove invaluable when attempting to generate therapeutic, cell replacement treatments. The objectives of this dissertation are to:

1. investigate the proliferative and phenotypic response of cells within the adult retina and adjacent structures to selective retinal injury (Chapter 2);
2. explore the means by which endogenous neural precursors can be activated via exposure to exogenously applied, mitogenic growth factors (Chapter 3);
3. direct the fate of progeny generated following mitogenic activation *in vivo* (Chapter 3); and
4. isolate neural precursors and investigate their behavior *in vitro* (Chapter 4).

Collectively, the results of these studies provide understanding into how endogenous, adult retinal precursors can be activated, and provide insight into their progenitor-like potential in a therapeutic context.

CHAPTER 2: Proliferation and Expression of Progenitor and Mature Retinal Phenotypes in the Adult Mammalian Ciliary Body Following Retinal Ganglion Cell Injury

Preface and Significance to the Thesis

The adult mammalian eye fails to retain active, cell replacement activity following pathological events. This lack of adult neurogenesis remains despite the presence of endogenous neural precursors residing within the adult oculum that display a robust capacity for neuron production *in vitro*. In other areas of the CNS, injury to surrounding neural tissue can elicit a limited neurogenic response by resident neural precursors. The response of adult neural precursors to selective RGC damage in the mammalian retina, however, has not been reported. In this chapter, I examine whether ON axotomy, which results in the selective apoptotic death of RGCs in a predicatable and time-dependent manner, is sufficient to induce the proliferation and/or up-regulation of cellular machinery in the CB that is responsible for neuron production by RPCs during development.

Preliminary results of this work have been presented in abstract for at the 33rd Annual Meeting of the Society for Neuroscience (2003) by Nickerson P, Emsley J, Myers T.L., Clarke D.B., "Proliferation and nestin expression in the injured and uninjured adult mammalian retina". I acknowledge the significant intellectual contribution by Dr. Jason Emsley (Harvard Medical School, Boston, MA) who was instrumental in experimental design and evaluation of our hypotheses. I would also like to thank Tanya Myers (former Technician in the Clarke lab) for teaching me the surgical techniques required for this work, and for significant surgical contributions to the study. Finally, Dr. Clarke has

meticulously edited this chapter and has provided valuable insight into the interpretation of my results.

Introduction

The vertebrate retina originates from a population of RPCs in the embryonic primordium of the diencephalon. During retinogenesis, a temporally choreographed sequence of progenitor proliferation and subsequent induction of retinal cell phenotypes is initiated. These distinct cellular phenotypes, which will comprise all 7 neural cell types of the adult retina, emerge in a well characterized histogenic order (Young, 1985; Marquardt, 2003). Throughout adulthood, the neurogenic capacity of the vertebrate eye is attenuated and, in the case of the mammalian eye, is virtually abolished. Continually active post-natal and adult RPCs have been described in the lower vertebrate eye, including cells located at the border of the retina and adjacent epithelium termed the ciliary margin (CM) of the amphibian eye (Raymond and Hitchcock, 1997), and retinal pigmented epithelium (RPE), rods and Müller glia in fish (Reh and Levine, 1998). Cells originating from the CM of the lower vertebrate eye continuously generate a population of retinal neurons throughout metamorphosis and adulthood (Fernald, 1990). These progenitors thus contribute to the prodigious capacity for neural regeneration in many lower vertebrates. Some higher non-mammalian vertebrates, such as the post-natal chicken, also retain the capacity to repopulate the adult retina throughout adulthood and under pathological conditions (Fischer and Reh, 2000b; Fischer and Reh, 2001).

In mammalian systems, the existence of adult retinal neurogenic potential was demonstrated using an *in vitro* colony-forming (neurosphere) assay (Tropepe et al., 2000; Ahmad et al., 2000). This research identified a small population of nestin-positive cells

derived from the pigmented ciliary epithelium (CE), a bilayer of cells which cover the ciliary body (CB), a structure involved in mediating lens shape and the production of aqueous humor. These nestin expressing cells retain the ability to clonally proliferate, generate sphere colonies and self-renew for many passages. When exposed to differentiating media conditions, these cells generate progeny with phenotypes reminiscent of retinal neurons and glia. These CE cells, termed retinal stem cells (RSC), have been identified in rodent and human eyes (Tropepe et al., 2000; Ahmad et al., 2000). RSCs differ from many other neural precursors in that they can proliferate in the absence of mitogenic factors *in vitro*, and are not abolished following the genetic deletion of GFAP during development (Tropepe et al., 2000; Morshead et al., 2003b). *In vitro*, these cells exhibit multipotentiality and express transcription factors (Pax6, Six3, Chx10, Rx, Lhx2) and phenotypes associated with other general precursors in the central nervous system (Nestin, Musashi1, SSEA-1) (Ahmad et al., 2004b). *In vivo*, mammalian RSCs reside in a quiescent state showing no proliferative activity under control conditions. This quiescence, coupled with the absence of a putative marker, have challenged our ability to characterize and determine a role for endogenous RSCs.

The *in vivo* response of cells in the adult mammalian CB and CM to changes in their intrinsic gene expression has also been investigated. Mutant mice undergoing constitutive activation of the canonical sonic hedgehog signaling pathway display enriched populations of proliferating cells in the adult CM (Moshiri and Reh, 2004). Cross breeding of these mutants with a model of retinal degeneration, a pro23his rhodopsin mutant, generated CM-derived divided cells having phenotypes consistent with

neurons and photoreceptors. This evidence suggests that adult CM progenitors can be stimulated to contribute to the repopulation of the adult mammalian retina.

The responses of adult mammalian CM and CE cells to injury have not been fully characterized. Injury to CNS tissue has been shown to generate changes in the local microenvironment via the release of diffusible factors and proteins mediating cell-cell interactions (Garcia and Vecino, 2003; Krueger-Naug et al., 2002; Krueger-Naug et al., 2003). Neurogenic regions in brain respond aggressively to pathology brought on by a wide range of damaging stimuli (Willis et al., 1976; Weinstein et al., 1996; Szele and Chesselet, 1996; Gould and Tanapat, 1997; Magavi et al., 2000). In lower vertebrates, a robust neurogenic response in the CM is elicited following injury to teleost (Raymond and Hitchcock, 1997) and chicken (Fischer and Reh, 2000a) retinas. To a lesser extent, adult mammalian Müller glial cells have been shown to re-enter the cell cycle and undergo reactive gliosis in response to excitotoxic lesions of the retina (Dyer and Cepko, 2000), up-regulate appropriate progenitor machinery when exposed to exogenous growth factors (Nickerson et al., 2005) and in some instances generate neuron-like progeny *in vivo* (Ooto et al., 2004) and *in vitro* (Das et al., 2006). The influence of specific classes of retinal cells on the activity of RPCs during development, including aspects of proliferation and the fate of cells generated by RPCs, has also been described. Of specific interest is the well characterized regulation of fate determination during early retinogenesis by accumulating numbers of RGCs (Gonzalez-Hoyuela et al., 2001; Wang et al., 2005). RGCs are a source of diffusible factors which act to regulate progenitor proliferation (Wang et al., 2005), and the further production of RGCs. Recent evidence suggests that the proliferative capacity of RPCs is also partially dependent on the

presence of newly generated RGCs (Mu et al., 2005). The influence of RGCs on the activity of quiescent neural precursors in the adult mammalian CB, however, has not been described.

The goals of this study were to examine the following in the adult rodent: levels of proliferation within the CB, CM and adjacent retina; the phenotype of proliferating cells and their progeny; and whether the response of these cells is altered following RGC injury. I describe a novel proliferative and phenotypic response of cells of the CB to optic nerve (ON) transection, an effect which is initiated before and increases during the period of RGC death. Taken together, my results demonstrate that quiescent progenitors can be activated following selective injury of at least one class of retinal neuron.

Methods

Subjects

Balb-c mice: The use of these albino mice eliminates pigment autofluorescence and, therefore, allows for fluorescent immunohistochemical phenotyping in the CB. Thirty-day old female *Balb-c* mice (Charles River, St Constant, Quebec) were housed 2 per cage in a colony vivarium maintained on a 12:12 hr light/dark cycle at constant temperature (21 °C) and humidity (40-50%). Food and water were available *ad libitum*. All animals were cared for by Dalhousie University animal care, following standards described by the Canadian Council for Animal Care, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Optic Nerve Transection

The following methods for ON transection were adapted from those described in rats (Mansour-Robaey et al., 1994;Clarke et al., 1998). Briefly, the left ON was

transected with micro-scissors approximately 0.5 mm from the posterior aspect of the sclera. Sham animals underwent the same surgical procedures without transection of the ON. Preservation of the blood supply was confirmed by microscopic examination of the retina through the dilated pupil. The right eye of each animal served as an internal, uninjured control.

BrdU Labeling of Proliferating Cells and Their Progeny

Proliferating cells were labeled during the S-phase of mitosis by administration of 5-bromo 2`-deoxy-uridine (BrdU)(Taupin, 2007). To detect proliferation during both the early and later time periods following injury, two labeling paradigms were used (Figure 1). In a "pulse" group, three daily intraperitoneal injections of BrdU (0.5 ml, 50 mg/kg, a dosage shown to consistently label proliferating cells in the CNS (Cameron and McKay, 2001;Hayes and Nowakowski, 2002) were performed for 5-days beginning on the day of surgery to detect early post-injury cell division. A second "chase" group received BrdU in their drinking water (1.5 mg/ml) from the time of injury until sacrificed. Water consumption was recorded for individual animals to control for differences in BrdU availability. Analysis of water consumption showed no significant differences between individuals and groups (not shown).

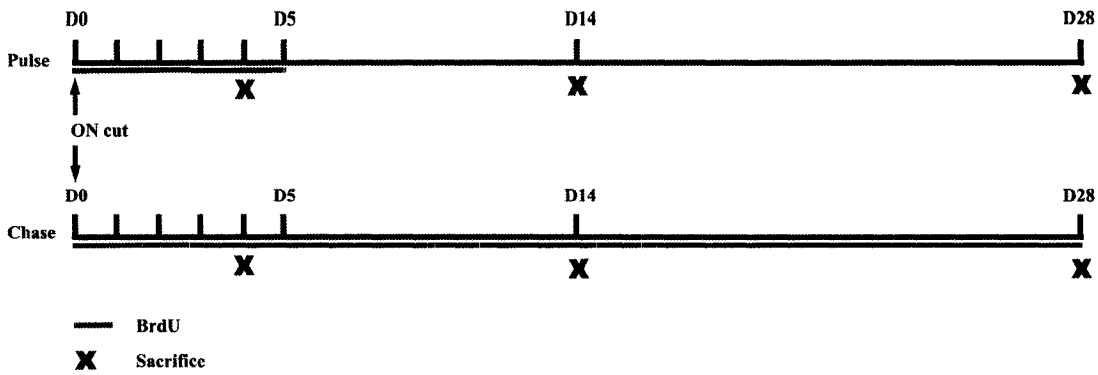


Figure 2-1. Experimental paradigm. BrdU lineage tracing: animals received either a pulse or chase BrdU labeling regimen to determine the time-course of proliferative responses to RGC axotomy. Following transection of the ON, animals were exposed to BrdU (RED line) and sacrificed (BLUE X) 4, 14 or 28-days following injury. Eyes were then examined immunocytochemically to assay the phenotype of dividing cells.

Table 2.1: Antibodies used in Chapter 2

Antibody	Cellular phenotype	Source and immunizing information
CRALBP	Adult Müller glia	1:1000, abcam, Cambridge MA, cat. Number ab15051, monoclonal clone B2, IgG2a
GFAP	Activated Müller glia and non-retinal astrocytes	1:100, Novo Castra, Newcastle UK, cat. number NCL_GFAP_GA5, clone GA5, species and isotype mouse-anti GFAP IgG1
Glutamine Synthetase	Adult Müller glia	1:1000, Chemicon, Temecula CA, cat. number MAB302, species and isotype mouse anti-GS IgG_2. Glutamine synthetase whole protein (1-373 bp) was used as the immunizing antigen
Nestin	Neuroectodermal, stem/progenitors, RPCs and activated Müller glia	1:500, BD PharMingen, San Diego CA, cat. number 556309, clone 401, species and isotype mouse anti-nestin IgG_1
Musashi-1	Neuroectodermal, stem/progenitors and RPCs	1:1000, Chemicon, Temecula CA, cat. number AB5977, species is rabbit anti-musashi-1
Pax6	RPCs, amacrine, horizontal and RGCs	1:500, Covance Research, Berkeley CA, cat. number PRB-278P, species is rabbit anti-Pax6
Chx10	RPCs, bipolar and a small sub-population of Müller glia	1:500, Chemicon, Temecula, CA, USA, cat. numbers AB9014 & AB9016, species is sheep anti-recombinant human Chx10
Factor 8	Vascular endothelia	1:500, Oncogene Research Products, San Diego, CA, USA, cat. number PC313, species is rabbit anti-factor 8 related antigen/vWF(Ab-1)
Doublecortin (DCX)	Immature, post-mitotic neurons	1:500, Chemicon, Temecula, CA, USA, cat. number AB5910, species is guinea pig anti-DCX

Antibody	Cellular phenotype	Source and immunizing information
ß-III tubulin (Tuj1)	Immature and mature neurons	1:1000, Chemicon, Temecula, CA, mouse anti-Tuj1
NeuN	Postmitotic CNS neurons	1:1000, Chemicon, Temecula, CA, USA, cat. number MAB377, species is mouse anti-NeuN
MAP-2	Mature CNS neurons	1:500, Chemicon, Temecula, CA, USA, cat. number AB5622, species is rabbit anti-MAP-2
Recoverin	Photoreceptors and bipolar cells	1:4000, Chemicon, Temecula, CA, USA, cat. number AB5585, species is rabbit anti-recoverin
BrdU	Anti-5-bromo 2`-deoxy-uridine	1:1000, Research Diagnostics Inc., Flanders NJ, USA, Sheep anti BrdU

Sacrifice and Retinal Tissue Processing

Animals were sacrificed 4, 14 and 28-days following surgery (n=4-5 per group). Animals were anesthetized using an intraperitoneal injection of a lethal dose of sodium pentobarbital (100 mg/kg) and perfused transcardially with chilled solutions of 0.1M phosphate buffer followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Prior to removal of the eyes, sutures were placed in the conjunctiva as a reference point for retinal orientation. Both eyes and brain were removed, post-fixed for 3 hours in 4% paraformaldehyde and cryoprotected in 30% sucrose. Eyes were embedded in gelatin and the same post-fix and cryoprotection procedure was repeated before sectioning (35 µm) on a freezing microtome. Brains, which were used to confirm CNS bioavailability of BrdU, were removed, post-fixed in 4% paraformaldehyde and cryoprotected in 30% sucrose before sectioning at 40 µm. Sections were stored in Milonig's solution (0.1M PBS/0.075% sodium azide) until staining.

Immunocytochemistry

Cellular proliferation and phenotype were determined by fluorescent double and triple-label immunocytochemistry. Tissue was washed in 0.1 M PB for 5 minutes, briefly dipped in ddH_2O, and placed in 2N HCL for 2 hours at room temperature. After washing, sections were placed in blocking solution (8% serum, 0.3% BSA in 0.1 M PBS/3% Triton-X 100) for 1 hour at 4 °C. Sections were placed in diluent (3% serum in 0.1 M PBS/3% Triton-X 100) containing primary antibody against BrdU alone, or multi-labeled using two or more primary antibodies (see Table 1). Sections were then rinsed and incubated in either Cyanine®-conjugated or Alexa® fluorescent secondary antibodies for 1 hour at room temperature. Sections were mounted onto gelatin coated glass slides and dried. Vectashield® (Vector Laboratories) fluorescent mounting medium was used for Alexa 488 stained sections whereas Citifluor® (Marivac Limited) fluorescent mounting medium was used for sections stained using Cy2. All antibodies were tested using positive control tissue and primary antibody omission. Nuclei within sections were counter stained using Topro-3®-iodide (Molecular Probes, Eugene, Oregon).

Confocal Microscopy

Labeled sections were imaged using a Zeiss LSM 510 (or 510 META) confocal microscope. Plan-Apochromat 1.4 oil/DIC objective lenses ranging from 40-100X magnification were used. Pinhole diameter was maintained at 1.0-2.0 Airy units for all wavelengths when quantifying double and triple-labeled cells. Laser outputs were set at 5% (488nm), 80% (543nm) and 9% (633nm). Emission filters were 505-530nm (Cy2), 560-615nm (Cy3) and > 650nm (Cy5). Orthogonal analysis was used to ensure co-localization of all multi-labeled sections.

Cell Counting and Statistics

We used an n of at least 4 animals per group for our experiments. For BrdU labeling experiments, 3 sagittal sections at the level of the ON per animal were visualized on a Leica DM400 fluorescence microscope equipped with a Ludl electronic stepper stage. BrdU-positive nuclei within the CE, CM, and adjacent neural retina were counted and plotted using Neurolucida, Stereoinvestigator (Microbrightfield Systems). All counting was performed blind to the experimental conditions. Unused sections at the level of and adjacent to the ON were used for further phenotypic analysis.

BrdU data are expressed as the mean number of BrdU-positive nuclei per retinal section, at the level of the ON +/- 1.0 standard error of the mean (S.E.M.). A repeated-measures analysis of variance was performed, using the independent variable of surgical Group (3 levels: transected ON, non-transected ON (right eyes) and sham operated). A repeated-measures analysis allowed us to use right eyes as an internal control for each subject. When faced with a significant main effect, *Scheffe* and t-test *post-hoc* analyses were performed to test differences among individual groups.

To analyze changes in the number of Chx10-positive nuclei in the CE of animals across all groups, we used an analysis of covariance (ANCOVA), with the total number of cells in the CE (counts from CE at either end of the retinal section are pooled) of each section as the co-variate. This analysis was used to control for differences in the total number of nuclei within the CE of different sections. Chx10 data are expressed as the mean number of Chx10-positive cells +/- S.E.M., per retinal section. *t-test* post-hoc comparisons were used to compare differences among groups following a significant main effect.

Results

The Ciliary Body and Epithelium Contain Two Distinct Populations of Nestin-Positive Cells in the Uninjured Eye

Nestin is an intermediate filament protein previously identified in adult neural stem/progenitor cells, embryonic neuroectodermal stem cells (Lendahl et al., 1990), reactive adult astrocytes and radial glia following pathology (Clarke et al., 1994), RSCs and progenitors (Sheedlo and Turner, 1996), neurogenic Müller glia (Fischer et al., 2005a) and in floating spheres generated from the CE of adult mice (Tropepe et al., 2000;Ahmad et al., 2000). Consistent with these *in vitro* reports, immunocytochemical staining of retinas in our *in vivo* study revealed a population of nestin-positive cells in the CB (Figure 2b-d). Nestin-positive cells are present in epithelium extending from the peripheral edge of the retina and RPE into the CB (Figure 2c). More sparsely distributed, single nestin immunoreactive cells are present in the CE adjacent to the vitreous (Figure 2b), which is consistent with the non-pigmented layer of the CE. These cells radiate 1 or 2 thin processes deep to the inner CE, nestin immunoreactive cell population. Double-label immunocytochemistry revealed that nestin-positive cells of the CB do not express fibrillary acidic protein (GFAP), CRALBP or glutamine synthetase (data not shown), lending further evidence in support of previous reports that CB stem/progenitor cells do not possess a glial phenotype (Morshead et al., 2003a).

A previous report indicates that periendothelial cells and pericytes of CNS vasculature express nestin (Alliot et al., 1999). To determine whether nestin-positive cells in the CB are associated with vasculature, we double-labeled for nestin and endothelial-specific anti-factor-8 (Figure 2c-d). Double-labeling with anti-factor-8 revealed two distinct populations of nestin-positive cells. In the deep layers of the CB, nestin co-

localized with factor-8-positive blood vessels (Figure 2c-d), corresponding to pericytes and/or periendothelial cells (high magnification in Figure 2d, arrowhead) and not luminal endothelia (Figure 2d, arrows). Nestin-positive cells in the CE (including individual "displaced" cells as well as those that form a chain that is continuous with RPE), however, are distinct from these nestin/anti-factor-8-positive cells of the CB vasculature by their location within the superficial epithelium of the CB and the absence of factor-8 expression.

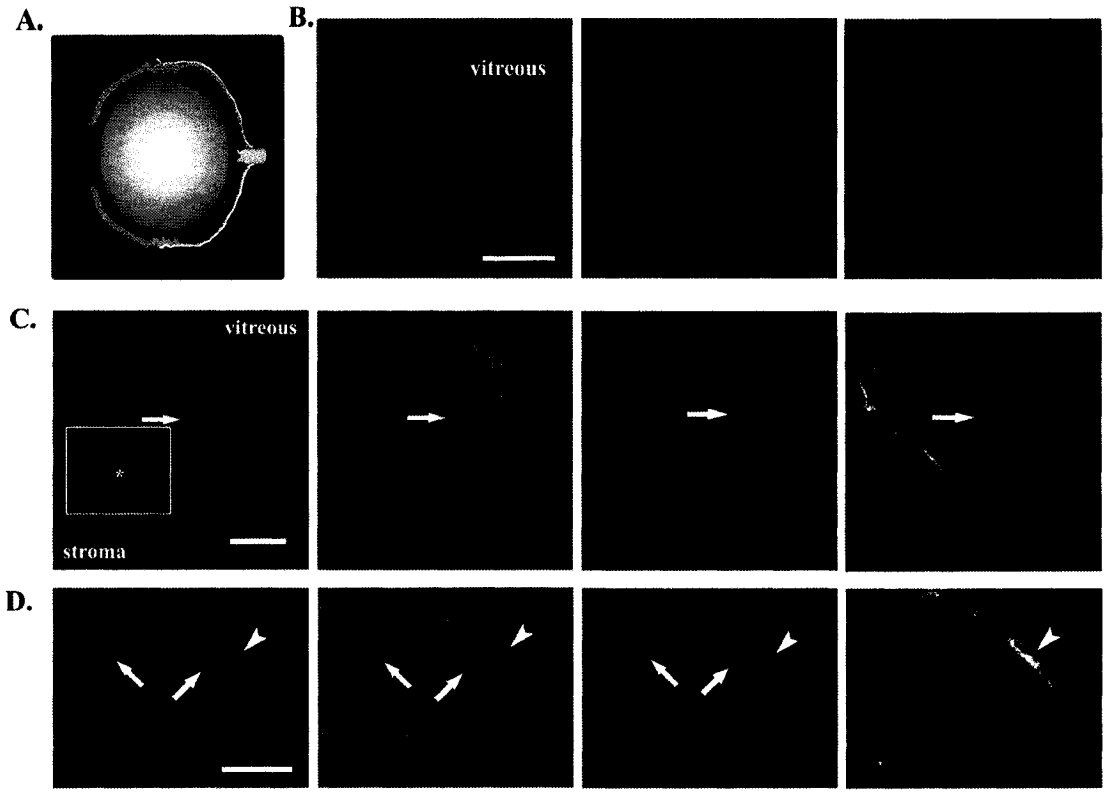


Figure 2-2. Two distinct populations of nestin expressing cells are present in the adult CB. In uninjured (or control) eyes, two populations of cells of the CB express nestin. (A) Schematic showing the location of the CB. Small square inset delineates the views represented in micrographs, with the pars plana to the right and iris toward the left B-D. Asterisk indicates a blood vessel. (B) Example of a single, nestin immunoreactive cell (Green) found in the superficial epithelial layer of the CB, adjacent to the vitreous. Scale bar = 20 μm. (C) Nestin-expressing cells in the superficial layer of the CB corresponding to the pigmented CE (arrows and as shown in B) do not stain for anti-factor 8. In contrast, some cells deep to the CE (outlined by insert and as shown at higher power in D) are co-immunoreactive for nestin (Green) and endothelial-specific anti-factor 8 (RED). Scale bar = 20 μm. (D) Higher magnification of insert in C reveals the presence of nestin on vascular pericytes and/or periendothelium (arrow heads), and not luminal endothelial cells (arrows). Scale bar = 10 μm. Topro-3 stained nuclei are BLUE in all micrographs.

Nestin-Positive Cells of the Ciliary Epithelium Undergo Low-Level Proliferation in Response to RGC Injury

Examination of retinas from both sham operated and right eye, non-injured controls revealed extremely low-level proliferation in the CB and CM, as seen by BrdU incorporation (typically 1or 2 nuclei per section). Cellular proliferation followed a temporal progression in both the injured and non-injured retinas. However, proliferation was significantly greater in injured vs. uninjured eyes at 4 and 14-days, when examined using a BrdU chase paradigm. Differences in proliferation over 14-28-days were not detectable following a pulse of BrdU, likely due to dilution of BrdU by subsequent cell division and/or death. Furthermore, BrdU administration restricted to the first few days following axotomy would not be seen due to a several-day delay in CB proliferative response to axotomy (Berkelaar et al., 1994). Due to these observations, data from animals exposed to chronic (chase paradigm, Figure 1) BrdU exposure are presented for the remainder of the study when assessing proliferation.

Interestingly, proliferation increases in the CB of non-injured eyes over time, suggesting that cells of the CB undergo a constitutive, slow rate of cell division and/or a population of cells recently generated outside of the eye migrate to the CB. However, the proliferative response following RGC injury is increased and is temporally restricted: a significant increase in the number of BrdU-positive nuclei in the CB is seen at 4-days ($\text{Mean}_{\text{INJURED}} = 1.67$, $\text{Mean}_{\text{UNINJURED}} = 0.17$, $p < .003$), peaks at 14-days ($\text{Mean}_{\text{INJURED}} = 10.9$, $\text{Mean}_{\text{UNINJURED}} = 2.0$, $p < .004$) and returns to levels that are not significantly different from controls by 28-days ($\text{Mean}_{\text{INJURED}} = 13.7$, $\text{Mean}_{\text{UNINJURED}} = 9.9$, $p > 0.05$) post-injury.

To determine whether cells of the CB and CE that proliferate in response to RGC injury express nestin, double-label immunocytochemistry was performed. Staining revealed a population of recently divided cells in the CE that expressed nestin following ON transection (Figure 3a). Orthogonal confocal analysis confirmed the presence of BrdU-positive nuclei within chains of nestin expressing cells, which themselves extend into the RPE (Figure 3b). These dividing cells, however, were restricted to the pars plicata and did not extend into the pars plana, suggesting that dividing progeny have not migrated into the CM or adjacent neural retina.

Orthogonal analysis was used to quantify the number of nestin, BrdU and nestin/BrdU-positive cells present in the CE within injured eyes (Figure 3d). At twenty-eight-days following injury, a time which corresponds with the highest numbers of BrdU-labeled nuclei, a mean of 58 +/- 9 cells per section were nestin-positive, of which 27% were BrdU-positive (16 +/- 6). Conversely, approximately 80% of the BrdU-positive cells in the CB at this time expressed nestin, indicating that the vast majority of recently dividing cells exhibit this progenitor-like phenotype.

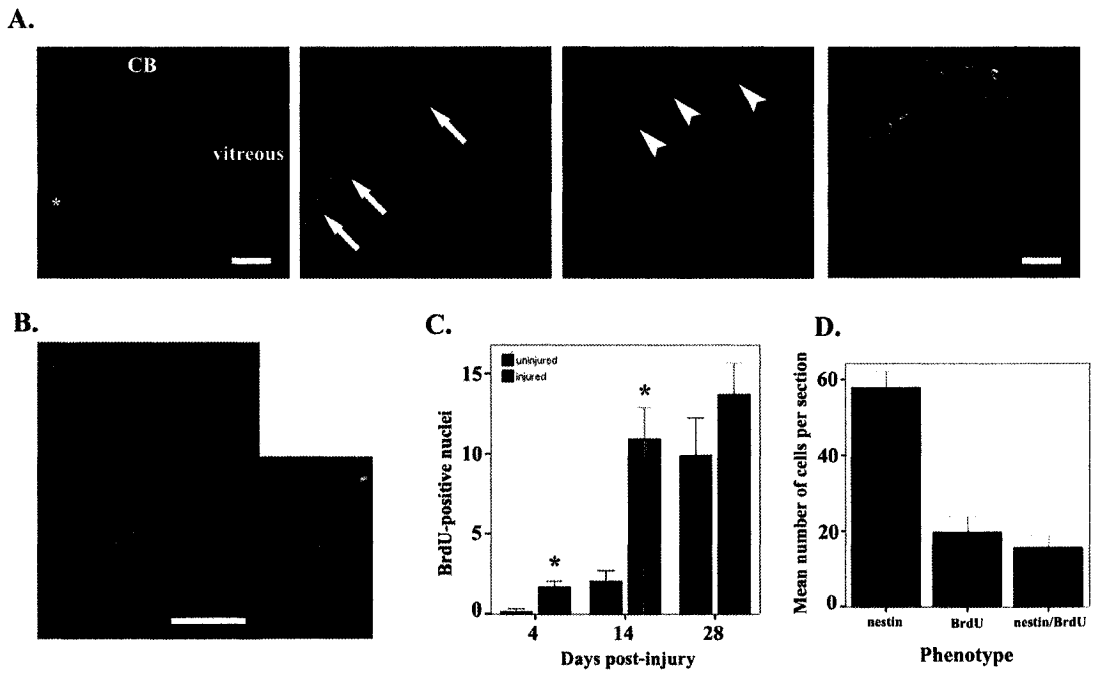


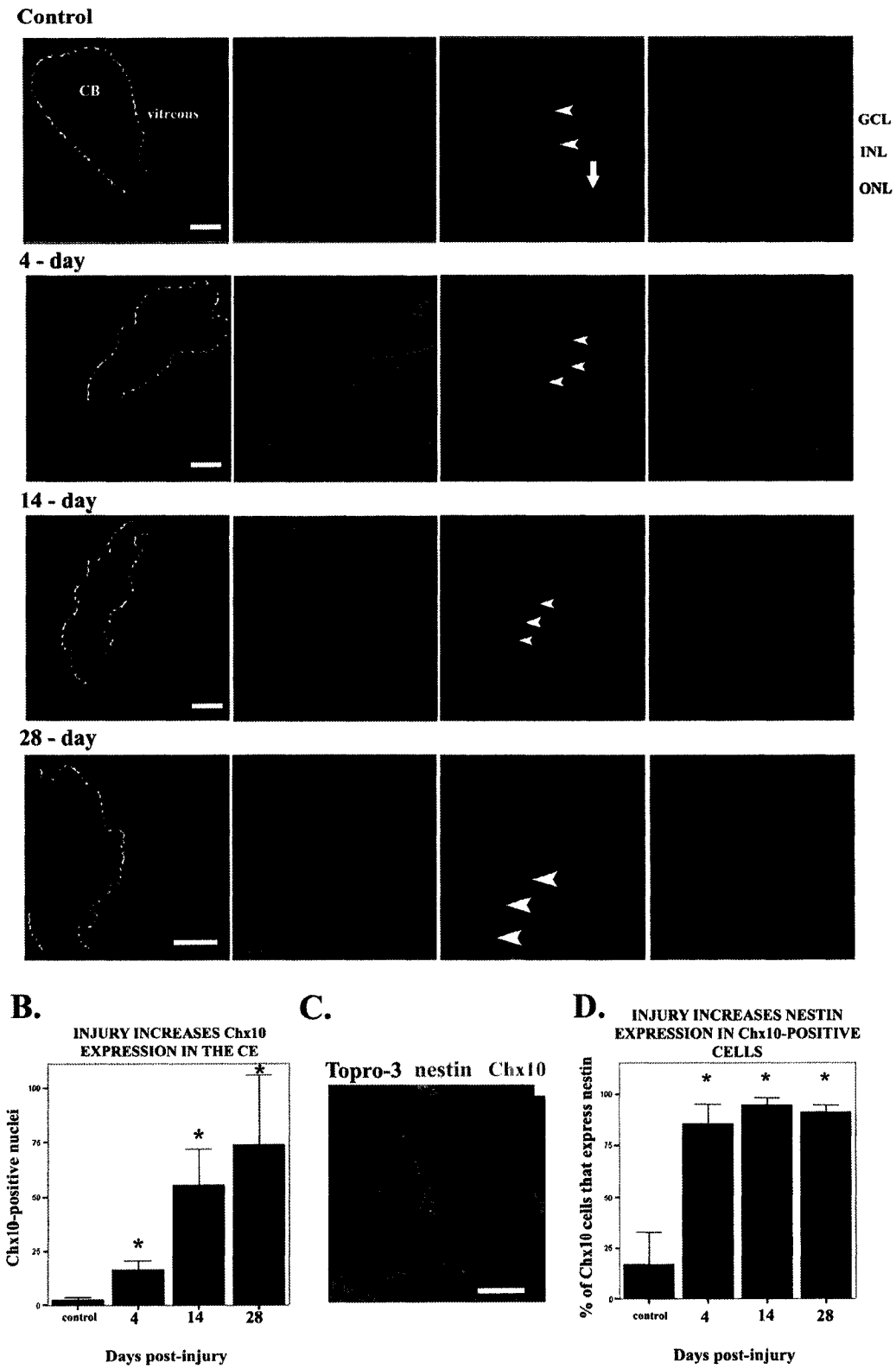
Figure 2-3. Nestin-positive cells in the CE proliferate in response to ON transection. (A) Nestin immunoreactive cells of the CE (GREEN) form a chain-like arrangement that is continuous with the RPE (arrows, asterisk delineates the end of the RPE). By 4 days post-injury, BrdU-positive nuclei (RED) emerge in the CE (arrowheads). Scale bar = 20 μm; example shown is 28 days after ON transection; Topro-3 positive nuclei are BLUE. (B) Orthogonal confocal analysis showing co-localization of BrdU-positive nuclei within a chain of nestin-positive cells. Scale bar = 10 μm. (C) A significant increase in BrdU labeling (mean number of BrdU-positive cells per section +/- S.E.M.) is seen at 4 and 14 days post-injury compared with controls. By 28 days post-injury, BrdU labeling in controls is statistically similar to injured eyes (* = significant between injured and uninjured eyes at $p < 0.05$). (D) By 28 days post-injury, the majority (approximately 80%) of BrdU-positive cells are nestin-positive, indicating that a sub-population of dividing cells in the CE expresses a progenitor phenotype.

Ciliary Epithelial Cells Up-Regulate Nestin and Chx10 in Response to Injury

To confirm that progenitor-like cells in the CB respond to retinal injury, we analyzed expression of the transcriptional regulators musashi-1, Pax6 and Chx10, all of which are phenotypes expressed in retinal stem cells and RPCs (Ahmad et al., 2004a). No cells in the CM or CB expressed musashi-1 or Pax6, with or without retinal injury (data not shown). Under control conditions, Chx10 expression was restricted to inner nuclear layer cells of the retina (Figure 4a, arrow), and only rarely were Chx10-positive cells found in the CB. Following ON transection, however, an increase in the number of cells expressing Chx10 was evident in the pars plana and pars plicata of the CB (arrowheads in injured sections). Analysis of cell counts indicated a significant increase in the number of Chx10-positive nuclei 4 (mean = 16, +/- 4, $p < 0.02$), 14 (mean = 55, +/- 16, $p < 0.02$) and 28 (mean = 74, +/- 32, $p < 0.05$) days following injury compared with controls (mean = 2.3, +/- 0.9) (Figure 4b).

Low magnification observations indicated that Chx10-positive nuclei reside in close proximity to nestin-positive cells. Orthogonal confocal analysis confirmed the co-expression of nestin and Chx10 in CE cells (Figure 4c). Counts revealed that the vast majority of nestin-positive cells expressed Chx10 in their nuclei 4 (85.4%), 14 (94.3%) and 28 (90.6%) days following injury ($p < 0.01$ or less) relative to controls (16%) (Figure 4d). These data indicate that two previously identified RSC phenotypes (nestin and Chx10) are up-regulated, and are expressed by cells in the CE following RGC injury.

Figure 2-4. Chx10 is up-regulated within nestin-positive cells in the CE following injury. (A) In control and sham-operated retinas (example in A), Chx10 (RED) immunoreactivity is restricted to inner nuclear layer cells of the retina (arrow for example; nuclei are BLUE; abbreviations are: outer nuclear layer ONL, inner nuclear layer INL and ganglion cell layer GCL; outline of CB in Topro-3 stained images) and no Chx10 staining is detected in the CB. At 4, 14 and 28 days following injury, Chx10-positive cells can be seen in the CE (arrowheads). Chx10-positive nuclei appear in close proximity to nestin immunoreactivity (GREEN). Scale bars = 50 μm. (B) Cell counts reveal a significant increase in the number (mean per retinal section) of Chx10-positive nuclei in the CE following injury. (C) High magnification, orthogonal analysis confirmed co-localization of Chx10-positive nuclei within nestin-positive cells. Scale bar = 20 μm. (D) Following injury, the proportion of Chx10-positive cells that also express nestin is increased relative to control. * in B and D = significant relative to control, $p < 0.05$.

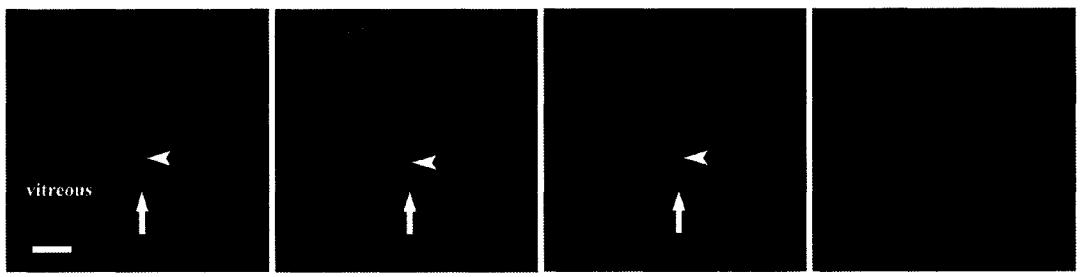

A.
Control
CB
vitreous
GCL
INL
ONL
4 - day
14 - day
28 - day
B.
INJURY INCREASES Chx10 EXPRESSION IN THE CE
Chx10-positive nuclei
control
4
14
28
Days post-injury
C.
Topro-3 nestin Chx10
D.
INJURY INCREASES NESTIN EXPRESSION IN Chx10-POSITIVE CELLS
% of Chx10 cells that express nestin
control
4
14
28
Days post-injury

Recoverin is Up-Regulated in Non-Dividing Cells of the CE Following RGC Axotomy

The observation that 20% of BrdU-positive cells in the CB were not nestin-positive (Figure 3d) prompted us to investigate whether newly-generated, terminally differentiated neuronal phenotypes were present. To examine this possibility, double-label immunocytochemistry for BrdU and either βIII-tubulin or doublecortin (DCX) was performed. Despite the presence of βIII-tubulin-positive cells in the CB (consistent with parasympathetic innervation), none of these cells was BrdU-positive. The absence of DCX labeling in all conditions suggests that no new neurons were generated. In support of these data, generic post-mitotic, mature neuronal phenotypes and RGCs (MAP-2, NF-200 & NeuN) were also negative (data not shown).

To determine whether BrdU-positive cells generated following injury resembled any remaining, specific retinal cells, immunocytochemistry against a select panel of retina-specific antibodies was performed (Pax6 for amacrine cells, PKCα for bipolar cells, GS for Müller glia, calbindin for horizontal cells and recoverin for photoreceptors and bipolar cells (Haverkamp and Wassle, 2000). Under control and uninjured conditions no cells, including newborn (BrdU-positive) cells, possessed these phenotypes (data not shown). In contrast, there was a small population of recoverin expressing cells (Figure 5a, arrow for example) seen at 14 and 28 days post-injury, suggesting that cells resembling photoreceptors or bipolar cells were present. However, none of these cells was BrdU-positive (Figure 5), suggesting that non-proliferative cells in the CE up-regulate recoverin following injury.

A.



B.

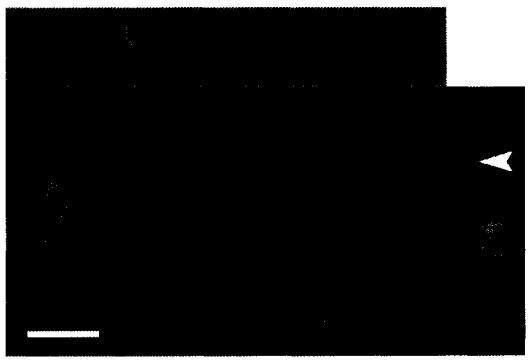


Figure 2-5. Recoverin immunoreactivity is increased in non-dividing cells of the CE following retinal injury. (A) Unlike in control animals, recoverin-positive cells (GREEN) appear in, and possibly deep to the bilayered CE, as early as 4-days following RGC injury (arrow for example). Recoverin labeling is intermixed with BrdU-positive nuclei (RED; arrowheads for examples). Scale bar = 20 μm. (B) High magnification, orthogonal analysis confirmed an absence of co-localization between recoverin (GREEN) and BrdU (RED, arrowhead) indicating that non-proliferative cells up-regulate recoverin in response to retinal injury. Scale bar = 10 μm, Topro-3 stained nuclei are BLUE.

Discussion

Injury-Induced Activation of Progenitor-Like Cells of the CB

Taken together, our results show that the selective injury of at least one retinal cell type, the RGC, induces the activation a small population of cells in the CB that resemble RPCs. Nestin expressing cells of the CB proliferate, and up-regulate Chx10, a transcription factor expressed in active RPCs and RSCs. Finally, non-proliferative cells present in the CB up-regulate recoverin, a calcium sensor protein expressed in photoreceptors and bipolar cells, following injury, suggesting an attempt at retinal cell production by endogenous precursors.

In this report, we describe a population of cells in the CB of adult mammalian mice that express proteins similar to those seen in retinal stem and progenitor cells previously described by *in vitro* methods. The influence of the adult retina on the activity of retinal stem/progenitor cells in the CB has been investigated. Previous reports in lower vertebrates show a salient influence on adult marginal progenitors located in the peripheral retina by glucagon expressing neurons (Fischer et al., 2005b). Similarly, the capacity for murine RPCs and Müller glia to enter the cell cycle has been shown to be influenced by retinal tissue (Close et al., 2005). These results, supported by those reported here, clearly show that even into adulthood, neural progenitors in the peripheral aspect of the adult eye retain the capacity to respond to cues provided by the injury or death of retinal neurons.

Previous studies have identified a number of secreted factors that are released in the retinal microenvironment in response to RGC injury and death (Garcia et al., 2003).

Possible mechanisms of release include recently characterized, indirect pathways by which Müller glia release a variety of neurotrophic factors in response to retinal damage (Harada et al., 2002). In addition, the release of bFGF and GDNF have been shown to be induced by signaling from infiltrating microglia, cells that are present and active during the process of RGC death (Harada et al., 2002; Thanos and Richter, 1993). The specific mechanism by which CE cells respond to RGC injury is still unclear. Recent evidence suggests that an increase in proliferation (as measured by Ki67 and CyclinD1 expression) within nestin-positive cells of the CB follows exogenous application of insulin and FGF-2 (Abdouh and Bernier, 2006), observations that are consistent with those reported in chick (Fischer et al., 2002a) and which support a role for growth factors in the regulation of RSC activity.

An unexpected finding evident in our data is the moderate proliferative response detected in retinas 4-days following ON transection. As discussed above, factors released as a function of cell death can elicit a mitogenic response by CNS precursors. This proliferative response at 4-days post-axotomy, however, precedes the onset of RGC death post-axotomy by one day (Murphy and Clarke, 2004), indicating that although the proliferative response in the CE may be augmented by RGC death, it is initiated by injury.

Of particular interest in our study is the activation of transcriptional machinery appropriate for progenitor activity following RGC injury. Chx10 has been shown to play a pivotal role in the development of the neural retina. Although not necessary for the genesis of all retinal cell types, mutations in Chx10 result in abnormal eye growth, including micropthalmia, cataracts and iris malformations (Burmeister et al., 1996; Ferda

et al., 2000). Furthermore, the absence of Chx10 expression in Chx10 orJ/orJ mice results in small eyes but with a several-fold increase in adult retinal stem cells (Tropepe et al., 2000; Coles et al., 2006), an effect thought to be attributable to the loss in negative regulators elicited by the diminished RPC cell population. The initial *in vitro* phenotypic screening during the discovery of RSCs demonstrated that these cells express nestin and Chx10, two keystone phenotypes of RPCs. Consistent with these findings, we report *in vivo* evidence of axotomy-induced activation of a population of cells expressing both nestin and Chx10 in the same anatomical location. Although this observation is not sufficient to conclude that RSCs are being activated, these results are consistent with previous reports.

One well characterized role of Chx10 is the maintenance of proliferation within pools of RPCs. Chx10 mutations result in premature depletion of RPCs and subsequent reduction in retinal volume (Burmeister et al., 1996). In our study, we observed a robust increase in Chx10 expression with a relatively modest increase in proliferation. Although we would hypothesize that Chx10 up-regulation should be coincident with proliferation within the CE, it is possible that its expression is not sufficient to elicit a robust response in the absence of other mitogenic genes such as Pax6, which is not up-regulated post-RGC injury.

Recoverin Expression in the Ciliary Body After Injury

We identified a population of cells in the CE that up-regulate the calcium-binding protein recoverin following retinal injury. In the neural retina, recoverin is expressed at relatively early stages of photoreceptor differentiation (Johnson et al., 2001) and in a sub-

population of bipolar cells (McGinnis et al., 1999). The emergence of recoverin-positive cells in the CE following injury suggests that an attempt is being made for the differentiation of retinal cell types. CM progenitors in mice can be induced to generate photoreceptor-like cells *in vivo* following the combined over-activation of sonic hedgehog signaling and the presence of retinal degeneration (Moshiri and Reh, 2004). Since the vast majority of recoverin-positive cells in the retina are photoreceptors, one initial speculation might be that an attempt is being made toward their genesis. However, previous findings also suggest that cells of the mature bovine CB express components of phototransduction (Ghosh et al., 2004), and retain the capacity to contract in response to light in the absence of neural pathways (Bito and Turansky, 1975; Kargacin and Detwiler, 1985). In addition, CE and iris tissues can be induced to generate photoreceptor-like cells *in vitro* in the absence of stem cell culture conditions (Haruta et al., 2001). In light of evidence supporting a phototransductive ability by CB tissue, it remains possible that RSCs in the ciliary epithelium may represent a residual pool from which young, photoreceptive CE cells may be generated. The specific events leading to the production of recoverin-positive cells following axotomy appears to involve its up-regulation in the absence of proliferation (Figure 5 a-b). This observation supports the idea that CE progenitors may themselves differentiate into a post-mitotic cell type independent of proliferation, an effect reported in SVZ neural precursors following growth factor treatment (Shingo et al., 2001). The attempted genesis of an alternate cell type, a bipolar neuron for example, is also possible since recoverin is present in bipolar cells (McGinnis et al., 1997).

Conclusions

Our results show that, *in vivo*, cells of the CB respond to axotomy with a relatively low level of proliferation that is initiated before, and increases during, the period of RGC death. In addition, RGC injury increases the number of cells expressing Chx10 within the CB, and the proportion of those Chx10-positive cells that express nestin. Finally, it is evident that cells of the CE have the capacity to express a phenotypic marker (i.e. recoverin) seen in retinal photoreceptors and bipolar neurons, a response that is not coincident with cell division. From these data, we conclude that cells express phenotypes reminiscent of RPCs and RSCs in response to RGC injury in a time-dependent manner concordant with the known temporal progression of RGC death. In addition, cells of the CE can be induced *in vivo* to express phenotypes normally seen in retinal neurons. Further understanding of the mechanisms underlying the activation of RSCs, and their responses to different pathological conditions, may provide important insight into the future development of cell replacement strategies for treating various retinal pathologies.

CHAPTER 3: The Effects of Epidermal Growth Factor and Erythropoietin on Proliferation and Müller Glial Phenotype in the Adult Mammalian Retina

Preface and Significance to the Thesis

Müller glia have gained popularity as candidates for cell replacement in the adult mammalian retina, and have been identified as *bonafide* NPCs. Previous studies in other regions of the CNS have identified a number of factors, both natural and synthetic, that stimulate cell division within adult NPCs. In addition, exgenously applied growth factors can be used to augment the activity of, and cell types produced by, RPCs. In this chapter, I describe the response of Müller glia to the exogenous, *in vivo* application EGF and EPO, both factors previously shown to be essential regulators of NPC functioning in the adult CNS, and in the development of the retina. I demonstrate that a single intravitreal injection of EGF is sufficient to induce the re-entry of these cells into the cell cycle, and induce a process of interkinetic nuclear migration, a functional phenotype exhibited by RPCs during development. The co-application of EGF and EPO alters the distribution of proliferating nuclei that migrate across layers, and serves to suppress the glial phenotype within newly generated progeny. Finally, EPO treatment is sufficient to induce the expression of transcriptional regulators and intermediate filament proteins expressed in RPCs. This thesis investigates the modalities of activation with respect to candidate populations of NPCs in the adult mammalian retina. These data provide further evidence that exogenous treatment with growth factors that have previously been linked with adult NPC activity, activate adult mammalian Müller glia, and that their response to activation

progresses in a way that supports the theory that these cells are residual and quiescent RPCs.

Preliminary results of the findings in this Chapter have been presented at the 34th and 35th Annual Meetings of the Society for Neuroscience in October 2004 and November 2005. This work was published in abstract form as Nickerson P, McLeod M, Myers T.L., Clarke D.B. (2004) "Effects of EGF & EPO administration of proliferation and neurogenesis in the adult mammalian retina" and as Nickerson P, McLeod M, Myers T.L., Clarke D.B. (2005) "Phenotypic analysis of proliferating Müller radial glia: effects of EGF and EPO exposure in the adult mammalian retina". I acknowledge significant contributions of Tanya Myers (a technician in the lab at the time of these experiments) for the intravitreal surgeries, as well as providing technical support for tissue preparation, Dr. Marcus McLeod for help in generating experimental design strategies, preparing growth factor cocktails for intravitreal injections, and providing significant intellectual contributions to the preparation of the manuscript, and my supervisor Dr. David Clarke for his efforts in all aspects of the development and completion of this project.

Introduction

The vertebrate retina originates from eye fields located in the neuroectoderm of the anterior neural plate, which forms the diencephalon in mammals (Isenmann et al., 2003; reviewed by Marquardt, 2003). As the mammalian retina matures, the retinogenic capacity of RPCs is dramatically attenuated and, in the case of the rodent retina, no new progeny are generated past post-natal day 12 (Young, 1985). During these later stages of post-natal development, RPCs rearrange their intrinsic signaling properties and subsequently alter their responsiveness to surrounding microenvironmental cues. This

process involves the down-regulation of progenitor genes such as Pax6 and Chx10, and the acquisition of more mature Müller glial phenotypes (reviewed by Marquardt, 2003; and Dyer, 2003). These changes in progenitor activity and cellular lineage restriction are thought to account for the abolished neurogenic activity seen in the adult rodent retina.

Recent findings suggest that two distinct and mature populations of cells in the adult mammalian eye, Müller glia (Ooto et al., 2004) and retinal stem cells located in the pigmented epithelium of the CB (Tropepe et al., 2000; Ahmad et al., 2000), retain a number of characteristic features reminiscent of early RPCs. Müller glia have been shown to exhibit precursor-like activity in a number of vertebrate species (Dyer and Cepko, 2000; Fischer and Reh, 2001; Engelhardt et al., 2004; Rowan and Cepko, 2004) resulting in the retention of varying degrees of retinogenic capacity throughout adulthood (reviewed by Hitchcock et al., 2004). In lower vertebrates such as chick, Müller glia can be stimulated to proliferate, generate neurons, more Müller glia and other progenitor-like cells (Fischer and Reh, 2001). Progenitor-like activity in mammalian adult retinas has been limited, although characteristic progenitor behaviors such as proliferation, interkinetic nuclear migration, production of cells resembling mature postmitotic neurons, and reactive gliosis have been described in rodent Müller glia following retinal pathology (Ooto et al., 2004). Nonetheless, controlled regulation of adult mammalian Müller cell proliferation and RPC-like activity following exogenous activation has not been described.

Determination of cell fate within the central nervous system (CNS) is closely linked to regulation of stem/progenitor cell division. Neural stem/progenitor cell proliferation can be induced and maintained via exposure to extracellular cues, including

mitogens such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Vescovi et al., 1993), factors that have proven to be vital in the identification and classification of neural multipotentiality (Gritti et al., 1996; Weiss et al., 1996; Reynolds and Weiss, 1992; Morshead et al., 1994). A wide range of diffusible factors have been reported to influence retinogenesis (Altshuler et al., 1993; Austin et al., 1995; Ezzeddine et al., 1997; Guillemot and Cepko, 1992; Kelley et al., 1994; Lillien, 1995; Neophytou et al., 1997; Pittack et al., 1997; Zhang and Yang, 2001a). EGF has been shown to influence proliferation of RPCs during development (Ahmad et al., 1998) and *in vitro* (Tropepe et al., 2000; Ahmad et al., 2000), and to act by binding to EGF receptors located on cultured rabbit (Scherer and Schnitzer, 1994) and rat (Roque et al., 1992) Müller glia. During later stages of retinogenesis, up-regulation of EGF receptors on RPCs results in growth factor responsiveness favoring EGF signaling (James et al., 2004). In the adult chick, *in vivo* intraocular injections of EGF and FGF2 induce proliferation of progenitors of the CB, and production of mature neuronal cells (Fischer and Reh, 2003a). One report describes the induction of proliferation within populations of RPCs and Müller glia by EGF during late stages of retinogenesis, both *in vivo* and *in vitro*, and this effect that can be potentiated by co-exposure with transforming growth factor-β (Close et al., 2005). Although these data support EGF as a mitogen in the developing and acutely post-mitotic retina, its action as a regulator of proliferation in adult mammalian retina has not been investigated.

Exogenous application of factors can also play a crucial role in promoting and regulating lineage restriction and subsequent differentiation into specific mature cell sub-types. Erythropoietin (EPO), a potent angiogenic and erythrogenic factor (Youssoufian et

al., 1993), has been shown to exert a lineage commitment influence on subventricular zone neuron production following exogenous exposure *in vitro* and *in vivo* (Shingo et al., 2001). Neuroprotective influences on damaged brain, spinal cord (Kilic et al., 2005; Gorio et al., 2002) and retina (Junk et al., 2002; Weishaupt et al., 2004; Grimm et al., 2002; Grimm et al., 2004; for review, see Sasaki, 2003), as well as the promotion of retinal neural outgrowth (Bocker-Meffert et al., 2002), have been observed using exogenous EPO treatments. EPO and it receptor (EPO-R) are widely expressed throughout the adult CNS within capillaries (Brines et al., 2000), astrocytes (Masuda et al., 1994) and neurons (Morishita et al., 1997). In addition, EPO-Rs are present in the human developing CNS, including retina (Juul et al., 1998). Recent reports show EPO-R localization on virtually all E8 neuroepithelial cells, and persistent localization on radial glia and neurons in the developing midbrain (Knabe et al., 2004) lending further evidence that EPO is an active regulator of neural development. Despite reports demonstrating the ability of exogenous EPO to influence adult brain neurogenesis, there have been no reports concerning its ability to influence the phenotype of dividing retinal cells and newly generated progeny in the adult retina.

The aim of this study was to investigate the effects of intraocular EGF or EPO exposure on adult rat retinal cell proliferation and phenotype. Furthermore, the effect of co-administration of EGF with EPO was examined to determine the possible influence of EPO upon phenotypes expressed by proliferating retinal cells. The findings of this study indicate that a single intravitreal EGF injection exerts a potent mitotic response in adult mammalian Müller glia. In addition, EGF treatment stimulates Müller glia to express phenotypes that have been reported in RPCs during retinal development. Finally, co-

treatment with EPO alters the phenotype of Müller glia seen following EGF-induced proliferation.

Methods

Subjects

Sixteen, 6-month old female Wistar rats (Charles River, St. Constant, Quebec) were housed 2 per cage in a colony vivarium maintained on a 12:12 hr light/dark cycle at constant temperature (21°C) and humidity (40-50%). Food and water were available *ad libitum*. All animals were cared for by Dalhousie University Animal Care following standards described by the Canadian Council for Animal Care. Experimental timeline is summarized in Figure 3-1.

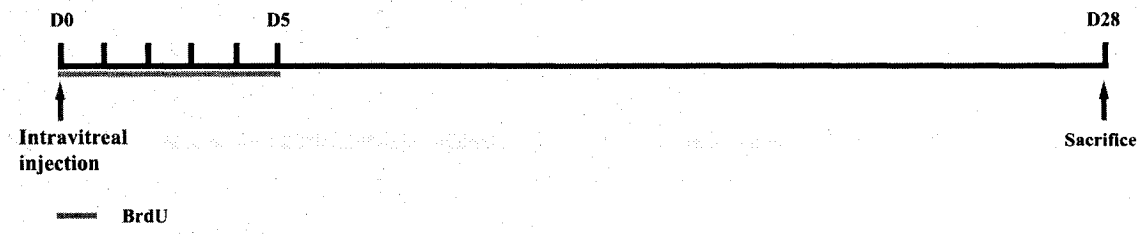


Figure 3-1. A schematic which outlines the 28 day experimental paradigm used in this study. On day 0 (D0), animals receive an intravitreal injection followed by 3, equally spaced BrdU injections over 5-days. Animals are then sacrificed at 28 days.

Intravitreal Injection

To assess the effects of mitogen exposure, and subsequent exposure to EPO in the retinal environment, intravitreal injections were performed as previously described (Mansour-Robaey et al., 1994; Clarke et al., 1998). Briefly, through a superior orbital approach, an incision was made through the sclera and retina using a 30-gauge needle. A single left posterior intravitreal injection (5µl) was performed using a 10µl Hamilton syringe adapted with a beveled glass pipette. The syringe was inserted into the vitreal space in such a way as to avoid damage to the lens and retinal vasculature. Following retraction of the syringe, the incision was sealed using Histocryl® adhesive and the incision closed. The right eye of each animal served as an internal control for the analysis. Experimental groups (n = 4 animals / group) received a 5 µl intravitreal injection of either vehicle (0.1% bovine serum albumin (BSA) in 0.1M phosphate buffered saline (PBS)), EGF (20 µg, recombinant human epidermal growth factor, Peprotech Canada Inc., Ottawa, Canada), EPO (30 International Units (IU), recombinant human erythropoietin, R&D Systems, Minneapolis, MN) or EGF/EPO combined (20 µg EGF + 30 IU EPO) in PBS-BSA vehicle solution.

Lineage Tracing of Proliferating Cells and Their Progeny

Labeling of cells during the S-phase of mitosis was achieved via the systemic administration of 5-bromo 2-deoxy-uridine (BrdU, 50 mg/kg). Three daily intraperitoneal injections were performed for 5 days following surgery. This dosage and regimen of administration has been shown to consistently label proliferating cells in the CNS (Hayes and Nowakowski, 2002; Cameron and McKay, 2001). Preliminary immunocytochemical assessment confirmed that BrdU labeling was restricted to the nucleus using Hoechst and

Topro-3 nuclear counter-stains (Invitrogen, Burlington, ON). BrdU staining in the subventricular zone and hippocampus of experimental animals served as positive controls.

Animal Sacrifice and Retinal Tissue Processing

At 28 days following surgery, animals were anesthetized using a single intraperitoneal injection of sodium pentobarbital (100 mg/kg) and perfused transcardially with chilled solutions of 0.1M PB followed by 4% paraformaldehyde (PFA) in 0.1M PB. Prior to removal of the eye, a single suture was placed in the conjunctiva as a reference point for retinal orientation. The lenses were removed and eyes were post-fixed for 3 hours in 4% PFA, cryoprotected in 30% sucrose for 24 hours and then embedded in 10% gelatin in 0.1M PB. The same post-fix and cryoprotection procedures were repeated for the gelatin-embedded tissue before sectioning (30μm) on a freezing microtome. Sections were stored in Millonig's buffer until staining. Brains used as positive controls were removed, post-fixed, cryoprotected and sectioned at 40 μm.

Immunocytochemistry

Cellular proliferation and phenotype within each treatment group were determined by double-label immunocytochemistry. Tissue was washed in 0.1M PB for 5 minutes, briefly dipped in ddH_2O, and placed in 2N HCL for 2 hours at room temperature. After washing, sections were placed in blocking solution (8% serum, 0.3% BSA in 0.1M PBS/0.3% Triton-X 100) for 1 hour at 4°C. Sections were placed in diluent (3% serum in 0.1M PBS/0.3% Triton-X 100) containing primary antibody against BrdU alone (1:1000, Research Diagnostics Inc, Flanders NJ, Sheep anti-BrdU), or multi-labeled

using two or more primary antibodies. Antibodies included: nestin (1:1000, BD PharMingen, San Diego CA, cat. number 556309, clone 401, species and isotype mouse anti-nestin IgG_1) that recognizes a 195 kDa band on western blot analysis; glial fibrillary acidic protein (1:100, Novo Castra, Newcastle UK, cat. number NCL_GFAP_GA5, clone GA5, species and isotype mouse-anti GFAP IgG1); glutamine synthetase (1:1000, Chemicon, Temecula CA, cat. number MAB302, species and isotype mouse anti-GS IgG_2. Glutamine synthetase whole protein (1-373 bp) was used as the immunizing antigen); and Pax6 (1:500, Covance Research, Berkeley CA, cat. number PRB-278P, species is rabbit anti-Pax6 generated against the peptide sequence QVPGSEPDMSQYWPRLQ). Sections were then rinsed and incubated in either Cyanine®-conjugated (Cy, BioCan Scientific, Mississauga ON) or Alexa (Invitrogen, Burlington ON) fluorescent secondary antibodies for 1 hour at room temperature. TO-PRO® -3 iodide (T-3605, Molecular Probes, Eugene Oregon) nuclear counter staining was used for BrdU confocal analyses. Sections were mounted onto gelatin coated glass slides and dried. Vectashield® (Vector Laboratories, Burlingame, CA) fluorescent mounting medium was used for Alexa 488 stained sections whereas Citifluor® (Marivac Limited) fluorescent mounting medium was used for sections stained using Cy2. Slides were coverslipped using 1 ounce, no. 0 micro cover glasses.

Confocal Microscopy

Multi-labeled sections were visualized using a Zeiss LSM 510 META confocal microscope. Plan-Apochromat 1.4 oil/DIC objective lenses ranging from 40-100X magnification were used. Pinhole diameter was maintained at 2.0 airy units for all wavelengths. Laser outputs were set at 5% (488nm), 80% (543nm) and 50% (633nm).

Emission filters, including band-pass filters, were used to control for channel bleed through as follows: long pass >633nm (Cy5), band-pass 505-530nm (Cy2) and band pass 560-615nm (Cy3). Orthogonal analysis was used to ensure co-localization of all double-labeled cells.

Cell Counting of BrdU-Labeled Nuclei

For BrdU labeling, 4 randomly chosen retinal sections oriented sagittally at the level of the optic disc were counted from each eye. Cell counts were performed in the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer nuclear layer (ONL), ciliary margin (CM) and CB. Unused sections at or adjacent to the optic disc were used for further phenotypic analyses. Sections were visualized on a Leica DM400 fluorescence microscope equipped with a Ludl electronic stepper stage. BrdU-positive nuclei within specific layers of the retina, as well as the CE, were plotted using Neurolucida, Stereoinvestigator (Microbrightfield Systems). Cell counts for double-label phenotypic analysis of BrdU-positive nuclei were acquired using confocal analysis. All counting was performed blind to the experimental conditions.

Statistical Analysis

Data were analyzed using SPSS 10 for Windows. Homogeneity of variance and normality tests were performed to ensure that the data met the requirements for parametric statistics. Counts of BrdU-positive nuclei were compared between experimental groups (vehicle, EGF, EPO, EGF/EPO) using 1- and 2-way analyses of variance (ANOVA) for main effects. Left (injected) and right (internal control) eyes within each animal were also included in the analysis. Scheffé and independent samples

t-tests were used for *post-hoc* comparisons when main effects were significant. Data are expressed as means ± the standard error of the mean (mean ± S.E.M.), per retinal section at the level of the optic disc.

Results

EGF-Treatment Induces Cell Proliferation in the Adult Mammalian Eye In Vivo

BrdU immunocytochemical and neurolucida analysis on retinas 4 weeks following exposure to an intravitreal injection of either vehicle or EPO revealed minimal proliferation as indicated by the near absence of BrdU-positive nuclei in these treatment groups (Figure 3-2A). In contrast, EGF and EGF/EPO-treated retinas displayed marked increases in BrdU-positive nuclei in various layers of the retina (Figure 3-2B). Significant increases in the number of BrdU-positive nuclei were seen in retinas following injection of EGF (684 +/- 147, $p < 0.05$) or EGF/EPO (838 +/- 94, $p < 0.01$) when compared with vehicle treatment (15 +/- 2.0). In contrast, no increase in the number of BrdU-positive nuclei was observed in either the EPO-treated group (17.3 +/- 3.8) relative to vehicle ($p > 0.05$), or in the EGF/EPO group relative to EGF alone ($p > 0.05$). The majority of BrdU-positive nuclei were observed to reside in the INL of both the EGF and EGF/EPO treated groups. A gradient of BrdU-positive labeling was observed within the EGF and EGF/EPO treated retinas, with the densities of BrdU-positive nuclei being greatest in proximity to the central retina (see plot, Figure 3-2A). The majority of sections examined revealed extensive BrdU-positive nuclear labeling along the entire central/peripheral axis of the retina; however, some sections had areas of absent labeling before reaching the peripheral tip. In addition, an absence of labeling in the outer retina (photoreceptor outer segments and the retinal pigmented epithelium) was consistent across all treatment

groups. Variability in the intensity of BrdU staining was also observed within retinal sections of EGF and EGF/EPO treated groups, suggesting that some cells could be dividing more than once.

Together, these data indicate that EGF exerts a potent mitogenic effect on cells in the adult mammalian eye, whereas no significant proliferative affect of EPO (alone or in combination) was observed.

EGF/EPO Treatment Induces a Re-Distribution of BrdU-Positive Nuclei Across Retinal Layers When Compared With EGF Alone

Our observation that EGF and EGF/EPO treated retinas contained significantly higher numbers of BrdU-positive nuclei (Figure 3-2B, above) prompted us to investigate potential differences in the distribution of dividing cells across specific retinal layers. Comparison of experimental groups revealed a significantly different distribution of BrdU-positive nuclei within specific retinal layers, as well as the CB and CM, when comparing EGF and EGF/EPO treatments. Specifically, increases in the number of BrdU-positive nuclei were observed in the GCL (182 +/- 71 vs. 73 +/- 32, $p < 0.05$) and CM (23 +/- 4.5 vs. 18.5 +/- 3.3, $p < 0.001$), while significant decreases in BrdU-positive nuclei were observed in the ONL (17 +/- 4.7 vs. 93 +/- 62, $p < 0.05$) and CB (36 +/- 4.7 vs. 64.5 +/- 21.2, $p < 0.05$) in EGF/EPO retinas relative to EGF alone (Figure 3-2C).

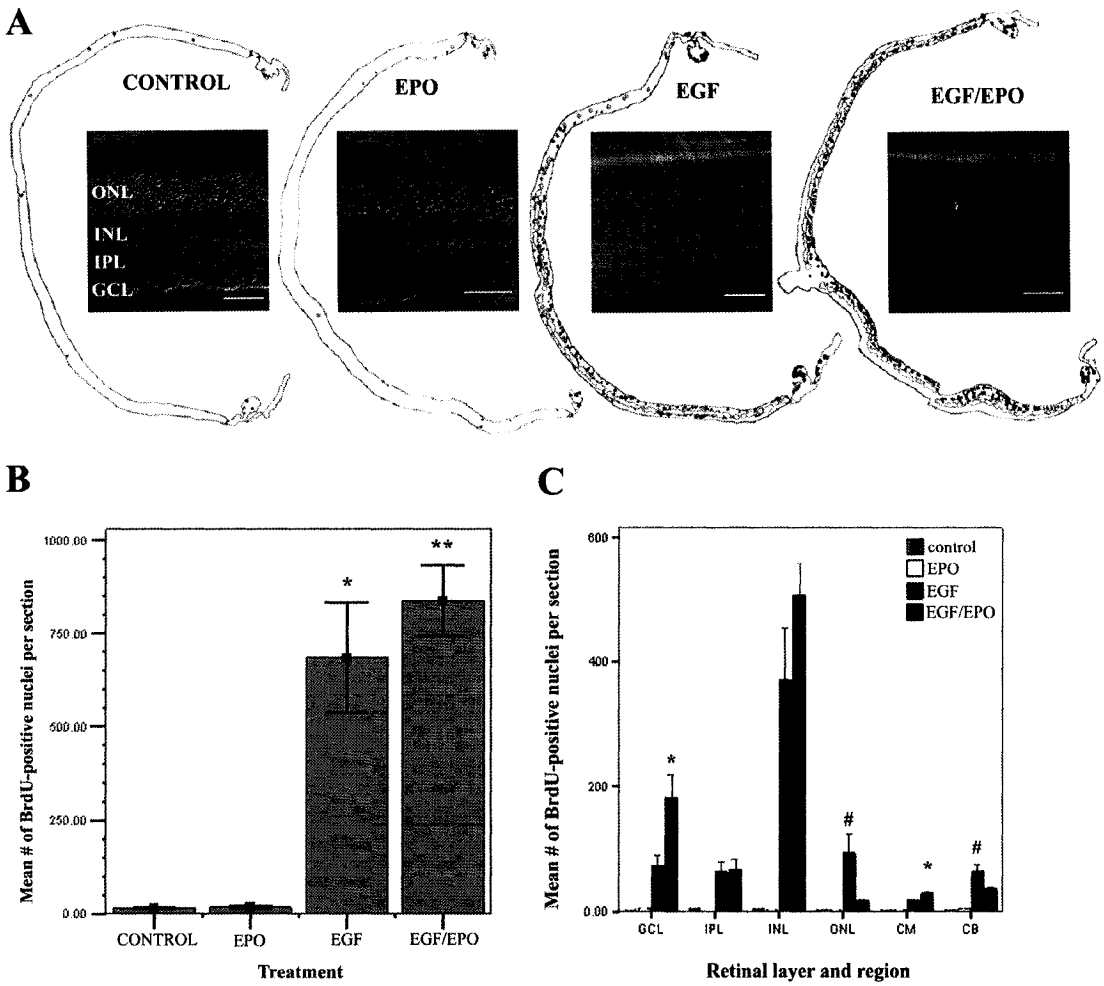


Figure 3-2. Retinal cell proliferation is increased 28 days following EGF treatment, but not by EPO treatment. (A) Representative plots of BrdU-positive nuclei within control, EPO, EGF and EGF/EPO treatment groups. Colors represent the distribution of BrdU-positive nuclei within specific retinal layers as follows: blue = GCL, green = IPL, yellow = INL, purple = ONL, red = CB & iris. Note the distribution of BrdU labeling throughout the central to peripheral axis of the retina. Insets are DIC images of single representative sections showing BrdU labeling (red) over nuclei (blue). Scale bars = 50 μm. (B) The number of BrdU-positive nuclei increases in retinas following EGF and EGF/EPO treatments relative to control retinas (* $p < 0.05$, ** $p < 0.01$). Treatment with EPO alone was not significantly different from control. (C) EGF/EPO treatment results in a re-distribution of BrdU labeling relative to EGF exposure alone, with increases in the GCL and CM, and decreases in the ONL and CB. EGF and EGF/EPO groups showed significantly greater numbers of BrdU-positive nuclei in all layers than in either control or EPO groups. Means represent the total number of BrdU-positive nuclei, per retinal section at the level of the optic disc; * in (C) represents significant increase relative to EGF; # represents significant increase relative to EGF/EPO.

EGF Induces Proliferation of Müller Glia

BrdU labeling experiments indicate that the greatest proportion of labeled nuclei resides in the INL following EGF exposure (Figure 3-2C). We aimed to test the hypothesis that Müller glia, resident cells of the INL (for review see Bringmann et al., 2006) that have been shown to display neural precursor activity in chick (Fischer and Reh, 2001), proliferate in response to EGF treatment. Double-labeling for glutamine synthetase (GS), a marker for postmitotic Müller glia, and BrdU was performed on control (Figure 3-3A), EPO (Figure 3-3B), EGF (Figure 3-3C) and EGF/EPO (Figure 3-3D) groups. Following EGF treatment, BrdU-positive nuclei co-localized within GS-positive Müller glia (mean number of double-labeled cells per 1mm of retina +/- S.E.M.) in the INL (32.9 +/- 5.8), as well as the GCL (3.7 +/- 1.3), IPL (1.8 +/- 1.2) and ONL (2.6 +/- 1.4) (Figure 3-4A). Double labeled cells in all layers other than the INL indicate the presence of ectopically positioned nuclei of recently dividing Müller glia, an observation consistent with interkinetic nuclear migration. Analysis indicated that EGF treated retinas displayed a significantly higher number of BrdU/GS-labeled cells ($p < 0.05$) relative to all other groups. Following EGF/EPO treatment, however, BrdU/GS cells were almost exclusively located in the INL (22.3 +/- 5.8). Analysis revealed a significant main effect of treatment ($p < 0.001$) and layer ($p < 0.001$) with no interaction ($p > 0.05$). *Post-hoc* comparisons indicated that EGF-treated retinas had significantly greater numbers of double-labeled cells in the GCL relative to EGF/EPO treated retinas ($p < 0.034$). No significant differences were seen when comparing EGF to EGF/EPO retinas in all other layers, despite overall higher means. Control and EPO groups were eliminated from statistical analysis due to the absence of proliferation in these retinas.

We next examined the proportion of BrdU-positive cells that also express GS, within EGF and EGF/EPO-treatment groups (Figure 3-4B). Analysis of all retinal layers together indicated that EGF treated retinas displayed a significantly higher proportion of BrdU labeled cells that express GS ($p < 0.042$), an effect consistent with the observation that no double-labeled cells were seen in the GCL, IPL and ONL of EGF/EPO-treated retinas. Analysis of the INL specifically revealed that a significantly greater proportion of BrdU/GS-positive cells were present in EGF-treated (32.9% +/- 15.5) retinas relative to the combination of EGF/EPO (22.2% +/- 11.7; $p < 0.001$).

Together, these data indicate that treatment of retinas with EGF induces proliferation of Müller glia, and initiates the ectopic displacement of their nuclei to various retinal layers that is consistent with interkinetic nuclear migration. In addition, compared with EGF treatment alone, combination-treatment with EGF/EPO significantly reduces the number of newly generated GS-positive cells and inhibits Müller glial interkinetic nuclear migration by restricting these BrdU-positive nuclei to the INL.

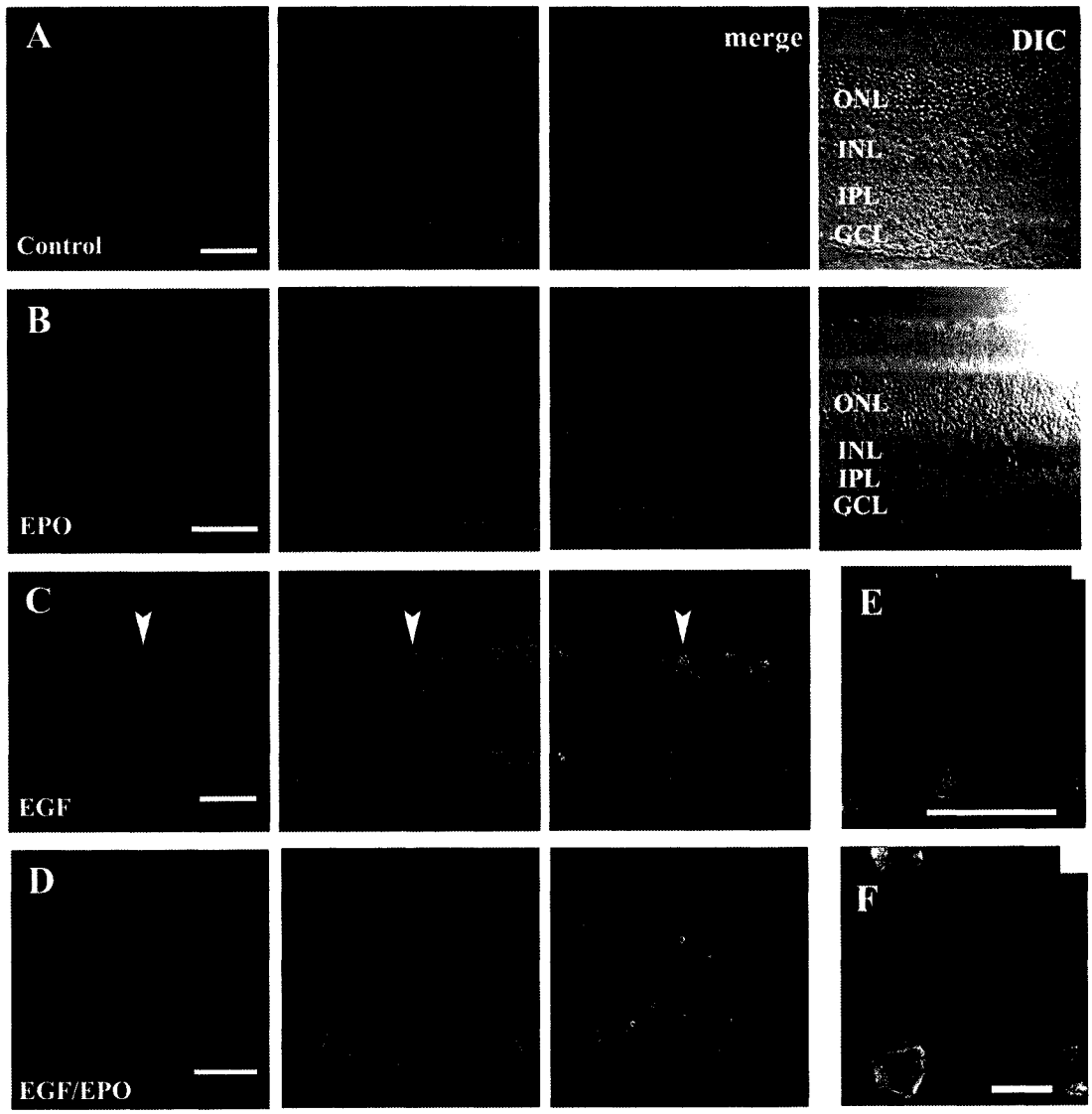


Figure 3-3. Müller glia proliferate in response to EGF treatment. GS staining (green) remained similar in control (A) and EPO (B) treated groups, with no proliferation indicated by the absence of BrdU-labeling (red). 28 days following EGF (C) and EGF/EPO (D) treatments, BrdU/GS double-labeled cells were seen predominantly in the INL (example highlighted by arrowheads). Orthogonal confocal analysis confirmed co-localization of BrdU and GS in EGF (E) and EGF/EPO (F) groups. Scale bars in A-E = 50 μm, scale bar in F = 10 μm, monochrome images are DIC.

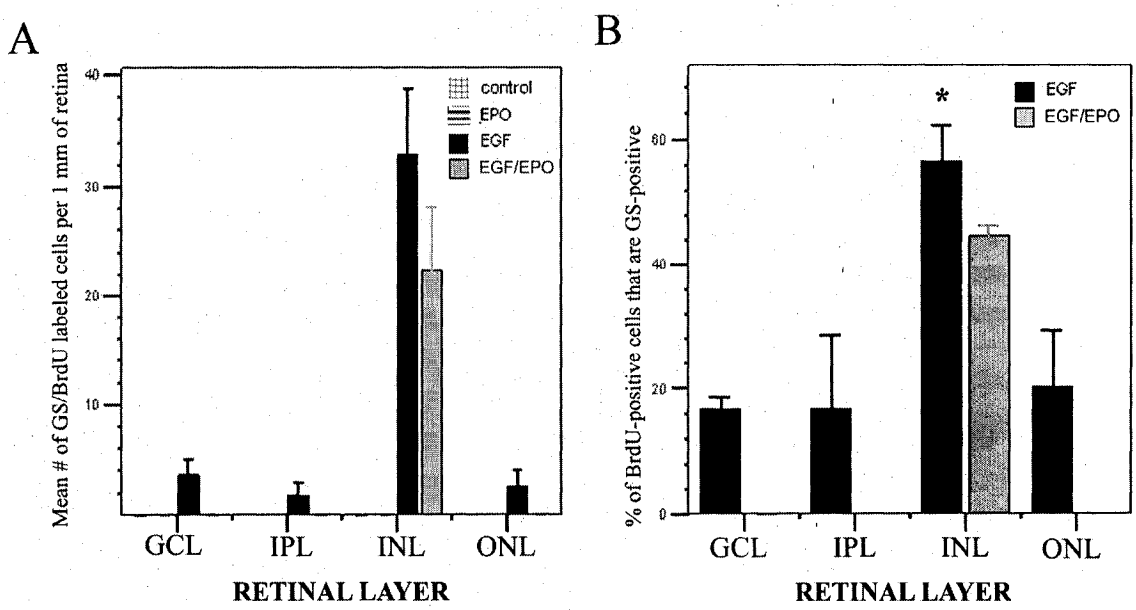


Figure 3-4. EGF treatment results in ectopically positioned BrdU-positive nuclei within cells that display a glial phenotype, while EPO co-treatment suppresses this effect. (A) Following EGF exposure, BrdU/GS double labeled cells are found in the GCL, IPL, INL and ONL. After EGF/EPO exposure, double labeled cells were restricted to the INL. (B) The proportion of BrdU-positive nuclei that co-localize within GS immunoreactive cells was compared across all retinal layers. In the INL where BrdU/GS double labeled cells are found in both treatment groups, EGF treatment resulted in a significantly higher proportion of double-labeled cells compared with EGF/EPO, * = $p < 0.001$).

EGF Induces GFAP Immunoreactivity in the Retina While EGF/EPO Reduces This Effect

To assess glial cell activation in the retina, GFAP immunocytochemistry was performed. In control and EPO treated retinas, GFAP immunoreactivity was restricted to the margin separating the vitreous and the GCL, corresponding to astrocytes (Figure 3-5A, large arrowheads), and to a small number of Müller glia adjacent to the peripheral retinal margin (not shown). Following EGF treatment, a robust increase in GFAP expression was present throughout the retina. The staining distribution and morphology were consistent with Müller glial cells. In addition, after EGF treatment small GFAP-positive Müller glial processes in various retinal layers were identified extending perpendicular to the normal Müller glial cell axis (Figure 3-5A, small arrowheads). Double-labeling for GS and GFAP confirmed GFAP localization on Müller glial processes in the IPL (Figure 3-5D, purple arrows). Only weak GFAP staining was seen in Müller cell bodies (Figure 3-5D, yellow arrows). In addition, astrocytes in the GCL and nerve fiber layer stained for GFAP but not for GS (Figure 3-5D, white arrows). Following EGF/EPO treatment, an overall reduction in GFAP expression was seen relative to EGF treatment alone. Remaining Müller glial GFAP immunoreactivity was restricted to processes corresponding to their end feet (Figure 3-5A, arrow). Counts (mean number of processes +/- S.E.M., per 0.25 mm of retina, sampled in the IPL) were performed to assess the number of GFAP-positive Müller glial processes in the IPL across all treatment groups (Figure 3-5C). Analysis revealed a significant main effect of treatment (ANOVA, $p \leq 0.036$), while *post hoc* comparisons confirmed that a significantly greater number of GFAP-positive processes reside in EGF (66.7 +/- 15.3) treated retinas relative to control (9.0 +/- 15.6, $p \leq 0.01$), EPO (18.2 +/- 28.8, $p \leq 0.017$),

but not in EGF/EPO (30.9 +/- 30.6, $p \leq 0.061$) treated retinas. These data indicate that EGF treatment results in increased GFAP immunoreactivity in Müller glia, while EPO treatment acts to suppress this effect.

EGF and EPO Independently Alter the Expression of the Developmental Markers Nestin, Pax6 and Chx10 in Müller Glia

We investigated changes in immunoreactivity for proteins associated with RPC activity typically seen during retinal development, in response to EGF and EPO treatments. Nestin is an intermediate filament protein localized in adult neural stem/progenitor cells, embryonic neuroectodermal stem cells (Lendahl et al., 1990), retinal stem cells and progenitors (Sheedlo and Turner, 1996) and, in some cases, reactive adult astrocyces and radial glia following CNS pathology (Clarke et al., 1994). There was a near absence of nestin staining in control and EPO treated retinas (Figure 3-5B), with the exception of low-level immunoreactivity for nestin observed on Müller glia located at the peripheral margin of the retina (not shown). Following EGF treatment, a robust increase in nestin expression was observed in Müller glia throughout the retina. In contrast, EGF/EPO treatment resulted in a reduction in nestin immunoreactivity to levels similar to controls and that differ only by the presence of lightly stained processes resembling Müller glia. Counts (mean number of processes +/- S.E.M., per 0.25 mm of retina, sampled in the IPL) were performed to assess potential changes in the number of nestin-positive Müller glial processes across all treatment groups (Figure 3-5C). Analysis revealed a significant main effect of treatment group ($p \leq 0.027$), while p*ost hoc* comparisons showed a significant increase in the number of processes following EGF treatment (50.7 +/- 8.5) relative to control (1.4 +/- 0.5, $p \leq 0.005$), EPO (15.3 +/- 26.8, p

≤ 0.019) and EGF/EPO (20.1 +/- 12.5, $p \leq 0.037$) treated retinas. These data demonstrate the ability of EGF and EPO to mobilize and suppress, respectively, the expression of nestin within adult Müller glia.

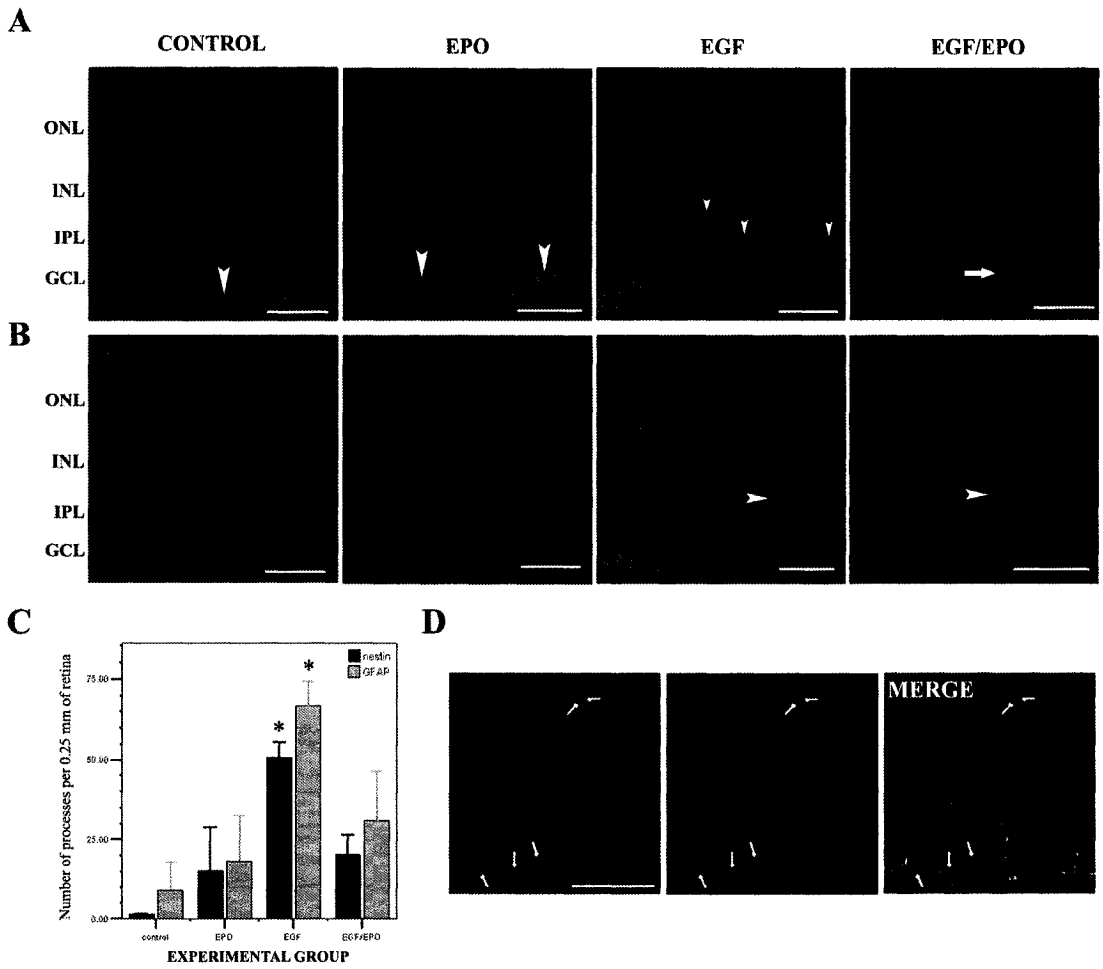


Figure 3-5 - The EGF-induced up-regulation of GFAP and nestin in Müller glia is suppressed by EPO (A) GFAP immunoreactivity (green) in retinas of control and EPO-treated animals is restricted to astrocytes of the nerve fiber layer (large arrowheads). Following EGF exposure, GFAP immunoreactivity increases substantially in Müller glia, and in small processes perpendicular to the main axis of the Müller cell (small arrowheads). Co-administration of EGF/EPO reduces GFAP immunoreactivity to near control levels. Arrow represents low-level GFAP persisting in Müller end feet. (B) Only following EGF exposure is nestin immunoreactivity seen in Müller glia (arrowhead) throughout the entire retina. Co-exposure to EGF/EPO returns nestin immunoreactivity to levels not different from controls. (C) The number of both GFAP and nestin immunoreactive processes were significantly greater in EGF-treated animals relative to all other groups. * = $p < 0.05$ relative to control. (D) Following EGF-treatment, double immunolabeling confirmed the expression of GFAP (RED) on GS-positive (GREEN) Müller cell processes and end feet (purple arrows for both). GS-positive Müller cell bodies of the INL are only weakly stained for GFAP (yellow arrows). Astrocytes in the GCL and nerve fiber layer stain for GFAP but not for GS (white arrows). Scale bars in A, B and D = 50 μm).

The homeodomain transcription factor Pax6 is expressed by mature amacrine cells located in the INL and GCL (de Melo et al., 2003), RGCs and horizontal cells (Zhang et al., 2003), as well as RPCs during development, and is required for RPC multipotentiality (Marquardt et al., 2001; Li et al., 2004). To determine whether Müller glia express phenotypes associated with RPCs, double-labeling for Pax6 and GS was performed. Orthogonal analysis revealed that Pax6 and GS are not co-localized under control conditions (not shown). Following EPO, EGF and EGF/EPO treatments, however, Pax6/GS-positive cells were identified in the INL only (Figure 3-6A). Cell counts were performed to assess the proportion of all Pax6 cells that were also GS-positive. Analysis revealed a significant treatment effect in the INL ($p \leq 0.002$), while *post hoc* analysis showed that a significantly greater percentage of Pax6-positive cells in the INL were GS-positive in the EPO (65.6 +/- 22.0) treated group relative to control (0.0 +/- 0.0, $p \leq 0.001$), EGF (17.4 +/- 1.1, $p \leq 0.004$) and EGF/EPO (25.1, +/- 1.9, $p \leq 0.011$) treatments (Figure 3-6B). These data indicate that EPO, and to a lesser extent EGF, up-regulate the pro-neuronal transcription factor Pax6 in adult Müller glia.

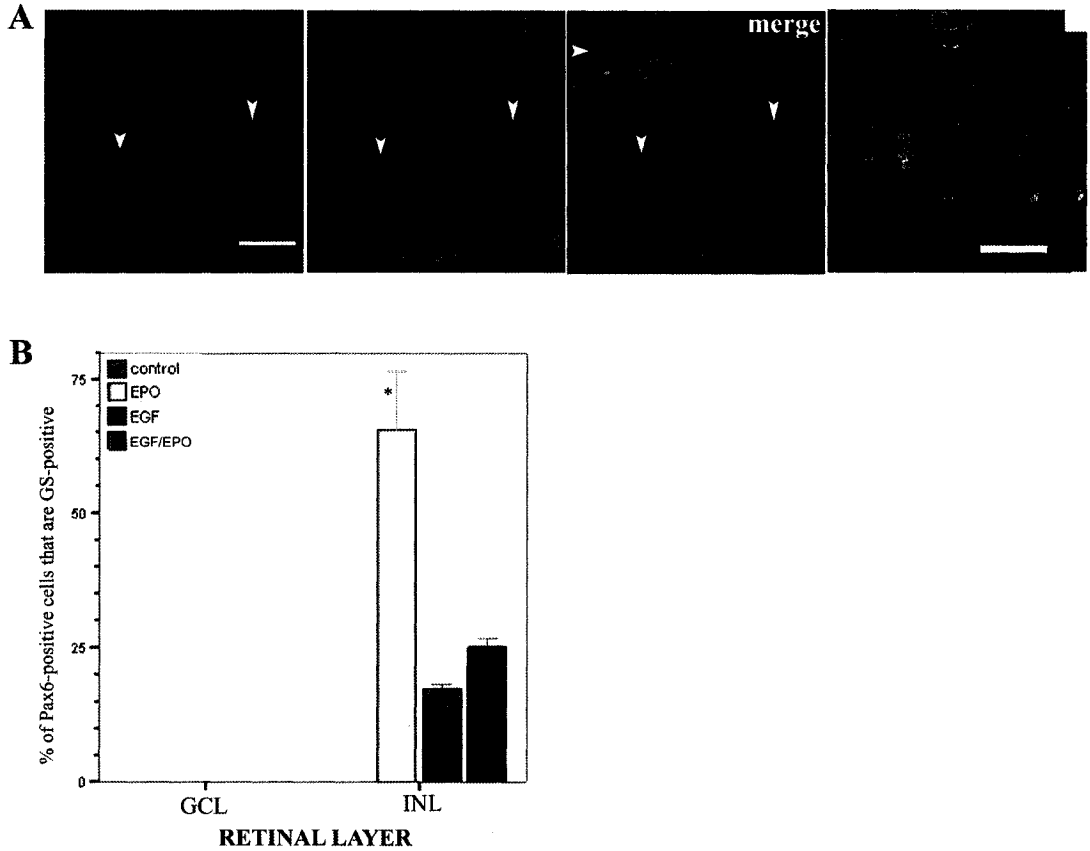


Figure 3-6 - EPO, and to a lesser extent EGF, up-regulate the transcription factor Pax6 in Müller glia

Under control conditions, Pax6-positive nuclei do not co-localize within GS-positive Müller glia (not shown). (A) 28 days following EPO (shown), EGF and EGF/EPO treatments, Pax6(red)/GS(green) double-labeled cells are present in the INL only (arrowheads show examples, inset is high magnification). Confocal (orthogonal) analysis was used to ensure co-localization. Scale bar = 50 μm for low magnification, 20 μm for orthogonal. (B) EPO-treated retinas contained the greatest number of Pax6-positive cells that were GS-positive compared with all other groups. * = significant relative to all other groups ($p < 0.01$).

We next examined the expression of Chx10, a RPC phenotype present in lower-vertebrate neurogenic Müller glia (Fischer and Reh, 2001), RPCs during development (Liu et al., 1994), bipolar neurons of the adult INL (Burmeister et al., 1996) and in a small population of adult murine Müller glia (Rowan and Cepko, 2004) within retinas from all treatment groups. Double-labeling for Chx10 and GS revealed only rare examples of Müller glia expressing Chx10 under control conditions. Following EGF and EGF/EPO treatments, however, Chx10/GS-positive cells were detected throughout the central retina (Figure 3-7A). In addition, Chx10-positive nuclei were seen in the ONL of retinas exposed to EGF, indicating that by 28 days post-treatment Chx10-positive nuclei may have migrated across retinal layers. No Chx10/GS labeled cells were seen, however, in retinas treated with EPO alone. Double-labeling for Chx10 and Pax6 was performed to assay for multiple phenotypes that co-localize within active RPCs during development. Although no co-localization was seen under control, EPO and EGF conditions, rare examples of Chx10/Pax6 double-labeled nuclei were detected within the peripheral retina following EGF/EPO treatment (Figure 3-7B).

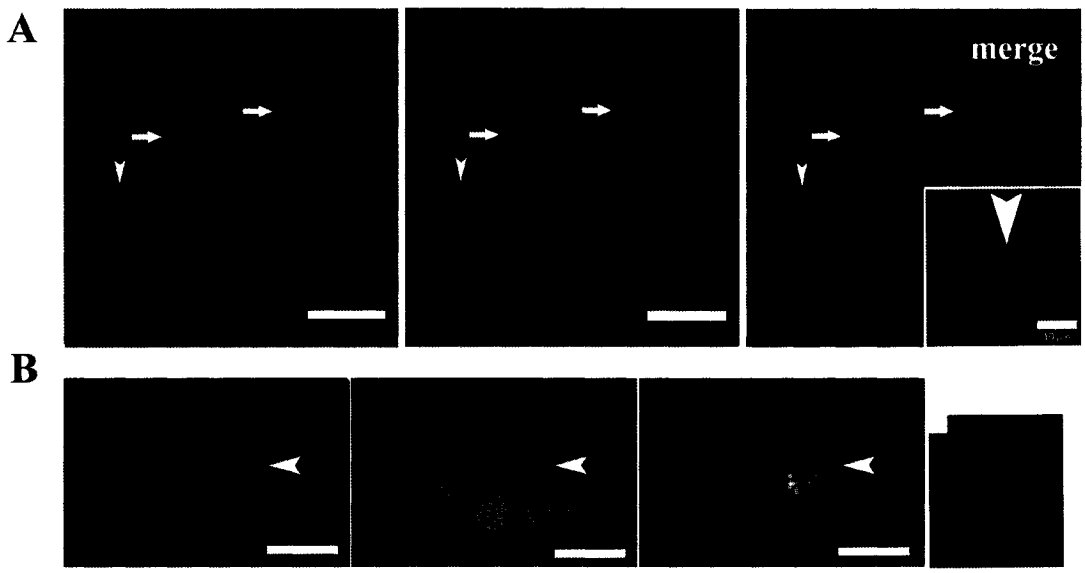


Figure 3-7 - Up-regulation of Chx10 in Müller glia is evident 28 days following exogenous activation

(A) Following EGF or EGF/EPO treatment (example from EGF treatment shown here), Chx10-positive nuclei (blue) are co-localized (arrowheads show example; inset for high magnification) within GS-positive Müller glia (red). Following EGF treatment, some Chx10-positive nuclei are ectopically located in the ONL (arrows). (B) 28 days following EGF/EPO treatment, a small number of Chx10-positive nuclei (red) are double-labeled (arrowhead) with Pax6 (green) in the INL near the periphery of the retina. Inset is orthogonal image. Scale bars in A = 50 μm (10 μm for inset); in B = 10 μm.

Discussion

Characterizing the regulatory mechanisms associated with proliferation and cell fate determination of Müller glia will provide important insight into retinal pathology and potential cell replacement strategies. It is evident from our experiments that a single intravitreal injection of EGF is sufficient to induce re-entry of Müller glia into the cell cycle. Furthermore, Müller glia and/or progeny derived from EGF-induced proliferation, survive and express phenotypes reminiscent of progenitor activity for at least several weeks. We have shown that intermediate filament proteins GFAP and nestin, as well as transcription factors Pax6 and Chx10, are up-regulated following growth factor exposure, observations that demonstrate the responsiveness of adult Müller glia to growth factor-treatment, and the possible de-differentiation of these cells into a progenitor-like state. In addition, treatment of adult retinas with EPO, a protein shown to exert both survival (Grimm et al., 2002; Grimm et al., 2004; Junk et al., 2002; Weishaupt et al., 2004) and neuronal lineage restriction influences upon CNS tissue (Shingo et al., 2001), influences the phenotype of recently proliferating Müller glia, and alters the retinal distribution of their nuclei. Finally, we show that EPO treatment is sufficient to up-regulate Pax6 in Müller glia, and restrict the Müller glial expression of GFAP and nestin, proteins that are up-regulated following EGF treatment.

EGF as a Mitogen in the Adult Mammalian Retina

In vitro, mammalian Müller glia have been shown to be responsive to EGF exposure via signaling through EGF receptors located on their cell surface (Scherer and Schnitzer, 1994). Activation of EGF signaling pathways initiate a robust proliferative response by these cells (Roque et al., 1992). The initiation of proliferation by mammalian

Müller glia *in vivo*, however, has been elicited predominantly via the induction of retinal excitotoxic pathology, such as that seen following intraocular NMDA exposure (Ooto et al., 2004). Previous attempts to induce Müller cell proliferation *in vivo* via the use of growth factor cocktails, including those containing EGF, have been only moderately successful (Ooto et al., 2004; Close et al., 2005). In contrast to the robust proliferative response elicited by the high-dose (20 μg) EGF regimen used in our study, a previous report (Ooto et al., 2004) using low-dose (100 ng) EGF exposure showed no significant increase in BrdU labeling. Differences in EGF dosage, tissue penetration, animal age, and BrdU labeling paradigms are all factors that may be responsible for these differing results.

Transcription Factor Expression Following Growth Factor Treatments

We demonstrate that EPO treatment is effective in up-regulating Pax6 expression in Müller glia, an observation that implicates EPO as a factor that may be capable of influencing the fate of newly generated cells derived from Müller glia. This effect is consistent with previously described mechanisms of EPO action within neural precursor cells present in the subventricular zone of brain. Specifically, the bHLH transcription factor Mash1 is up-regulated by EPO in subventricular zone progenitors (Shingo et al., 2001), indicating that EPO signaling occurs in tandem with the up-regulation of fate-influencing transcriptional machinery within known precursor cell populations. Previously, Pax6 expression in Müller glia has been correlated with the restriction of interkinetic nuclear migration within their cell bodies (Ooto et al., 2004). This functional phenotype was also evident in our experiments, wherein EGF/EPO induced Pax6

expression (Figure 3-6) correlates with a redistribution of BrdU-positive nuclei that is restricted to the INL, and not ectopic retinal layers (Figures 3-1 and 3-3).

Our efforts to determine whether Müller glial proliferation in the adult is associated with expression of transcription factors previously reported during retinal development led us to double immunocytochemically stain retinas for glutamine GS, a Müller cell marker, and Chx10, a potent regulator of RPC cell cycle (Burmeister et al., 1996). Whereas only rare examples of Müller glia that contained Chx10-positive nuclei were found under control conditions (perhaps reflecting a population of Chx10-positive Müller glia previously reported by (Rowan and Cepko, 2004), they were frequently identified in the INL following treatment with EGF/EPO (Figure 3-7A). The demonstration that fewer Chx10-positive than Pax6-positive Müller glia were seen following the same growth factor treatment likely reflects the exit of these cells from the cell cycle, an effect mediated by the extended period of time (28 days) between mitogen exposure and tissue examination.

Changes in Intermediate Filament Expression Following EGF and EGF/EPO Treatments

Plasticity of intermediate filament expression has been previously described in various classes of glia within the CNS (Frisen et al., 1995; Bignami and Dahl, 1979; Fitzgerald et al., 1990; Scherer and Schnitzer, 1991). Up-regulation of filamentous proteins such as GFAP, nestin and neurofilament within neurogenic Müller glia have also been described following exogenous activation in non-mammalian, vertebrate retinas (Fischer and Omar, 2005). Results from our study identify similar changes in Müller glial intermediate filament expression following EGF treatment. Increased Müller glial GFAP

and nestin expressions are evident in response to EGF treatment, indicating that cytoskeletal components within the cell are being mobilized. We show that the cellular selectivity of EGF and EPO action on filamentous protein expression is specific to Müller glia. For example, down regulation of GFAP in response to EGF/EPO treatment is selective to Müller glia, while its expression in non-retinal astrocytes remains unchanged (Figure 3-5). In addition, EPO treatment alone is insufficient in reducing constitutive GFAP expression in nerve fiber layer astrocytes. Together, these data indicate that the suppressive effect of EPO on GFAP is selective to activated Müller glia, and does not function to down-regulate GFAP in one other class of glial cell (retinal astrocyte) located in the retina.

We report similar expression patterns of nestin to those seen with GFAP in response to EGF and EGF/EPO treatments. It becomes difficult to conclude whether nestin expression is simply linked to general intermediate filament function, or whether its expression in Müller glia is an indicator of progenitor-like activity. Although nestin is present in a variety of neural precursors (Lendahl et al., 1990; Sheedlo and Turner, 1996; Ahmad et al., 2000; Tropepe et al., 2000), its identification in reactive astrocytes (Clarke et al., 1994) has diminished its acceptance as a selective progenitor phenotype. The avian homologue of nestin, transitin, has been shown to be selectively expressed in chick RPCs during development, CM progenitors in the adult, and is up-regulated in dividing, neurogenic Müller glia following exogenous growth factor exposure (Fischer and Omar, 2005). Consistent with the data presented here, nestin expression in the retina has been shown to correlate with RPC activity, and in tandem with the expression of other progenitor phenotypes such as Chx10 and Pax6 (Fischer and Reh, 2001; Fischer and

Omar, 2005). These observations are consistent with the idea that the expression of nestin under conditions including those described here, is consistent with Müller cell de-differentiation and subsequent induction of RPC-like potential.

A Subset of Newly Generated Cells May Fail to Differentiate

Some newly generated cells, identified via BrdU lineage tracing, did not stain for any phenotype tested. This observation could reflect either the mitotic activation of non-glioneuronal cells, or a delayed expression of mature cellular phenotypes, including GS, in newly generated cells. The genesis of new GS-negative Müller glia following EGF-induced Müller cell proliferation is probable, and would be consistent with previous observations indicating that GS expression in newly generated Müller cells can be delayed (Linser and Moscona, 1979). Furthermore, glucocorticoid receptor transcriptional activity has been shown to be suppressed during retinal proliferation (Linser et al., 1982; Grossman et al., 1994; reviewed by Vardimon et al., 1993). The failure of newly generated Müller glia to fully differentiate may explain the presence of GS-negative, BrdU-positive cells following EGF treatment, and is consistent with observations reported in chick wherein newly generated cells following NMDA treatment were negative for any phenotype tested (Fischer and Reh, 2001; Fischer and Omar, 2005).

CHAPTER 4: Neural Progenitor Potential in Cultured Müller Glia: Effects of Passaging and Exogenous Growth Factor Exposure

Preface and Significance to the Thesis

In chapter 3, I evaluated the response of adult mammalian Müller glia to exogenous activation, which included the use of mitogenic and morphogenic growth factors to direct their production of new retinal progeny. The limited progenitor-like response, however, prompted me to re-investigate Müller cell progenitor-like activity under isolated conditions. We established retinal cultures which allowed us to assess the influence of adult neurons on Müller cell proliferation, and then expose cultures to various growth factor regimens. Results derived from these studies confirmed that adult neurons exert a quiescence signal toward Müller glia, and that chronic maintenance *in vitro* permits their de-differentiation into a cell which resembles a RPC. In addition, our failure to generate neurons *in vivo* via exposure to neuron-promoting factors prompted us to test those factors *in vitro*. As a result, we confirmed that treatment of proliferating Müller glia with EPO and BDNF stimulates their differentiation into cells with phenotypic characteristics of neurons. Together, these data allow us to conclude that the RPC-like response by Müller glia *in vivo* is a precursor step toward the potential to generate neurons, as was seen *in vitro*.

Preliminary results of the findings in this Chapter have been presented at the 5th Forum of European Neurosciences (FENS) in July 2006. These results were published in Abstract form as Nickerson P.E.B., Clarke D.B. "*In vitro* analysis of proliferating Müller radial glia: expression of developmental phenotypes Pax6, nestin and Musashi-1" and have been submitted in abstract form to the 37th Annual Meeting of the Society for

Neuroscience as Nickerson P.E.B., Da Silva N. Myers T., Clarke D.B. "Growth factor responsiveness in cultured Müller glia: implications for neurogenesis in the adult mammalian retina". As well, I would like to acknowledge the significant contribution of Dr. Noel Da Silva in the Department of Physiology and Biophysics for the isolation and establishment of chronic Müller cell cultures and patch clamp electrophysiology, Kelly Stevens for RT-PCR analysis of CRALBP transcripts and Dr. Marcus McLeod for discussion on growth factor treatments.

Introduction

Lineage tracing evidence indicates that during the final phases of retinogenesis, a final cell division event produces one terminally differentiated retinal neuron and a postmitotic Müller cell (Turner and Cepko, 1987), the radial glial support cell of the retina. This direct lineage relationship between RPCs and Müller glia raises the question of whether postmitotic Müller cells retain progenitor potential into adulthood. Although Müller glia remain in a non-proliferative, quiescent state post-developmentally, their capacity to exhibit progenitor-like activity in response to a variety of stimuli has been demonstrated (Fischer and Reh, 2001; Ooto et al., 2004; Hitchcock et al., 2004). Specifically, Müller glia can be stimulated to re-enter the cell cycle and generate postmitotic, neuronal progeny in lower-vertebrates (Fischer and Reh, 2001; Wu et al., 2001) and in mammalian retinas, dividing Müller glia generate neuron-like progeny *in vivo* in response to excitotoxic injury and progeny-specific misexpression of pro-neural genes (Ooto et al., 2004). Recent work has identified stem cell like capacities, including self-renewal and multipotentcy, by mammalian Müller glia via the use of *in vitro* methods (Das et al., 2006). Together, these studies report a variety of functional and

phenotypic characteristics of Müller glia, which are akin those seen in neural stem/progenitors derived from other areas of the adult central nervous system (CNS).

The responsiveness of cultured Müller glia to a regimen of exogenously applied factors, however, is still widely unexplored. CNS stem/progenitor cells, commonly referred to as neural precursor cells (NPC), can be stimulated to proliferate into sphere colonies, clonally divide to self renew, and asymmetrically divide to generate a wide range of postmitotic progeny (Seaberg and van der Kooy, 2003). Extensive research has demonstrated the responsiveness of cultured NPCs to a selection of individual mitogenic and morphogenic compounds (Hagg, 2005). In particular, the use of different classes of growth factors has been shown to greatly increase the propensity toward neuron production by NPCs both in culture and *in vivo* (Hagg, 2005; Seaberg and van der Kooy, 2003). Many of these growth factors stimulate critical signaling that modulates aspects of cell cycle dynamics, as well as the fidelity of distinct cell types produced by NPCs during development. The discovery of the often pleiotropic actions of growth factors has generated an elaborate list of lineage-restricting substances. One such protein, erythropoietin (EPO), is an erythrogenic and angiogenic growth factor that, when exogenously applied either *in vivo* or *in vitro*, stimulates the production of neurons from NPCs derived from the adult CNS (Shingo et al., 2001). Recent *in vivo* work from our laboratory has demonstrated that exogenous EPO treatment up-regulates pro-neural, RPC-like phenotypes in adult rat Müller glia (Nickerson et al., 2005), suggesting that these cells may respond to growth factor exposure in a similar manner to that exhibited by NPCs. Exogenously applied brain-derived neurotrophic factor (BDNF) has also been shown to increase the number of neuronal progeny produced at the expense of glia in

populations of NPCs (Zigova et al., 1998; Louissaint, Jr. et al., 2002; Ahmed et al., 1995). Reports indicate that Müller glia express the high-affinity TrkB (Taylor et al., 2003), and low-affinity p75 neurotrophin receptors (Oku et al., 2002; Garcia et al., 2003), and that loss in p75 expression is implicated in the progression of retinoblastoma (Dimaras et al., 2006), a proliferative tumorogenic disorder. The possible influence of BDNF and EPO treatment on the differentiation, and progenitor-like behavior of proliferating Müller glia, however, has not been reported.

In this report, we isolate mammalian Müller glia and characterize the response of these cells to various *in vitro* media conditions. To examine the possible de-differentiation of Müller glia into progenitor-like cells, we compare the expression of RPC-like phenotypes by mammalian Müller glia derived from adult retinas during the initial culture phase, to those expressed by Müller glia that have been chronically maintained in culture (i.e. >25 passages). Our data indicate that Müller glia proliferate, up-regulate RPC phenotypes, and generate sphere-like colonies when in the presence of high-serum, expansion media conditions and reduced numbers of neurons. In addition, exposure of chronically maintained Müller glia to differentiating conditions, including those containing growth factors, results in the production of cells that bear immunocytochemical resemblance to developing and adult postmitotic retinal cells. Together, these data support recent evidence that at least some Müller glia are quiescent NPCs in the adult mammalian retina, and may provide a substrate for cell replacement therapy.

Methods

Retinal Dissection and Müller Cell Culture

Primary cultures of Müller glia from either postnatal day 15-20 C57B1/6 mice or adult, retired breeder female Wistar rats were established as described by Wang et al. (2003) with some modifications. All animals were cared for by Dalhousie University animal care, following standards described by the Canadian Council for Animal Care.

Rat retinal Cell Cultures

Retired breeder, female Wistar rats were anesthetized using a single intraperitoneal injection of ketamine hydrochloride (100 mg/kg), acepromazine (1.2 mg/kg) and Rompun (7.2 mg/kg) in 0.9% saline. Eyes were enucleated, punctured, placed in Hank's solution (Sigma, Canada) and maintained at 37°C during the dissection procedure. Lenses were removed and retinas were dissected from the eye cup. Care was taken to gently dissect the ciliary epithelium and optic disc away from the retina to avoid contamination by neural precursors previously identified in adjacent, non-retinal tissues (Tropepe et al., 2000; Ahmad et al., 2000). Retinas were then titrated and centrifuged at 1000 rpm for 10 minutes to separate retinal pigmented epithelium (RPE) from the retina. Remaining tissue was placed in 0.1% trypsin (Gibco, Canada) for 15 minutes at 37°C and mechanically dissociated. Cells were centrifuged, and re-suspended in Dulbeco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS, Sigma, Canada) and antimycotic mixture (penicillin 100 U/ml, streptomycin 100 µg/ml) and seeded on 100 mm culture dishes. Media was changed every 3 days without passaging.

Mouse Müller Cell Cultures

C57B1/6 mice were anesthetized, decapitated and enucleated. Eyes were incubated briefly for 60 minutes at 37°C in Hank's solution (Sigma, Canada) containing 0.1% trypsin (Gibco, Canada), 70U/ml collagenase type 2 (Worthington, Lakewood, USA) and antibiotic-antimycotic mixture (penicillin 100U/ml, streptomycin 100μg/ml, amphotercin 25μg/ml; Sigma, Canada). Retinas were gently dissected to avoid contamination with adjacent, non-retinal tissues. Cells were dissociated by trituration, centrifuged at 1000rpm for 10 minutes to separate RPE and plated onto 35-mm culture dishes containing DMEM with 10% fetal bovine serum (Sigma, Canada), and antibiotic-antimycotic mixture. Medium was left unchanged for 3 days and replenished every alternate day. Neuronal cells and debris were removed by gently washing in DMEM. After reaching full confluency, cells were passaged by plating onto culture dishes. Müller cells were identified by immunocytochemistry, RT-PCR and patch clamp electrophysiology (see below). Cells were maintained in 5mL flasks in DMEM with 10% FBS and 1% penicillin/streptomycin. Upon reaching confluency (approximately 4 days), cells were passaged by physically dissociating them from the culture flask using a pipette, and splitting them into additional flasks. This procedure was repeated for > 25 passages.

Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR)

RT-PCR methods and primers were adapted from (Mori et al., 2004). Briefly, chronically maintained Müller cell cultures were centrifuged, and total RNA was isolated using the TRIZOL (invitrogen, Canada) extraction kit according to manufacturer's instructions. DNA was digested using RQ1 RNase-free DNase (Promega, Canada), and remaining RNA was reverse transcribed using primers, oligo(dt)12-18 (Amersham Pharmacia Biotech, Canada) and moloney murine leukemia virus (M-MLV) reverse

transcriptase (Promega). PCR primer sequences used to recognize CRALBP transcripts were: 5'-CAAGAGGCAGTATGTCAGAC-3' and 5'-GAAGAGTTCAGGGTACTGGA-3'. Following a 1 minute denaturation at 94°C, material underwent PCR conditions as follows: 45 cycles of 30 seconds at 94°C, 1 min annealing at 53°C, 1 min extension at 72°C, and a final elongation of 10 min at 72°C. Amplified products were subjected to electrophoresis on a 2% agarose gel containing ethidium bromide. Gels were visualized using the Kodak Edas 290 (Kodak, Canada) under ultraviolet light.

Patch-Clamp Recordings

Whole cell currents from Müller cells were recorded using patch pipettes drawn from thin walled borosilicate capillary tubes (ID, 1.1-1.2mm; wall, 0.2mm, Micro-Hematocrit, Drummond Scientific Co., Bromall, PA) with resistances ranging from 5-10 MΩ. Pipette electrodes were filled with solution containing (in mM): 126 CsCl, 2 $CaCl_2$, 10 Hepes, 1 EGTA, 1 ATP, 0.1 GTP at pH 7.2. Current recordings were obtained using an Axopatch 200 patch-clamp amplifier (Axon Instruments, USA), and filtered at 1kHz and were digitized with an interfaced computer running BASIC-FASTLAB acquisition software (Indec Systems, Sunnyvale, USA). 100μM $BaCl_2$ was prepared prior to use in extracellular solution containing (in mM): 135 NaCl, 3 KCl, 2 $CaCl_2$, 10 Hepes, 11 Glucose, 1 Na_2HPO_4 at pH 7.2. Recordings were carried out at room temperature with solutions perfusing at 1ml per minute. Current-voltage (I-V) relations were measured in the last 10ms of each depolarizing pulse and plotted by using Microsoft Excel 2003 (USA).

Assessment of Proliferating Cells and Their Progeny In Vitro

Labeling of cells during the S-phase of mitosis was achieved via the addition of 5-Chloro-2`-deoxy-uridine (CldU, 0.01mg/ml, Sigma-Aldrich, St Louis, MO) to the culture media (see, Vega and Peterson, 2005). CldU was added to cultures for 4 hours before fixing, and immunocytochemical assessment using Hoechst and Topro-3 nuclear counter-stains (Invitrogen, Burlington, ON) confirmed that CldU labeling was restricted to the nucleus.

Growth Factor Treatment of Müller Cell Cultures

Chronically maintained Müller cell cultures were centrifuged, washed with DMEM, and re-suspended in priming media composed of: DMEM + 1% FBS + 1% penicillin/streptomycin + EPO (20 U/ml, recombinant human erythropoietin, Peprotech Canada Inc., Ottawa, Canada) and BDNF (50 ng/ml, Amgen Inc., Thousand Oaks, CA; a generous gift from Regeneron, Tarrytown, NY) for 3-hours. Cells were then washed and re-suspended in differentiation media containing EPO (0.1 U/ml in DMEM + 1% FBS + 1% penicillin/streptomycin) and BDNF (50 ng/ml in DMEM + 1% FBS + 1% penicillin/streptomycin) and plated down at a density of approximately 3.5 x 10^4 cells/ml onto uncoated 16-well chamber slides (Lab-tek). Cells belonging to the control condition did not receive growth factor priming (see above), and were plated down in growth factor free, differentiation media (DMEM + 1% FBS + 1% penicillin/streptomycin). Media was changed every second day, and cells were maintained for 7-days until fixation and preparation for immunocytochemistry.

Immunocytochemistry

Cultures:

Chamber slides containing cells were washed in chilled PB, fixed in 4% PFA for 15 minutes and washed. Cultures that underwent staining for thymidine analogue (CldU) incorporation were washed with 0.1M PB, briefly rinsed with ddH2O, and treated with 2N HCL for 15 minutes. Cultures that were not stained for CldU were not exposed to HCL treatment. Cultures were then washed and blocked in normal serum (8% serum in 0.1M PB/0.3% Triton-X 100) for 45 minutes at 4°C. Diluent (0.1 PB/0.3% Triton-X 100 with 3% serum) containing one or more antibodies (See Table 1) was added and incubated for 18 hours at 4°C.

Secondary Antibodies and Counter Stains

Cultures were incubated in either Cyanine®-conjugated (Cy; BioCan Scientific, Mississauga ON) or Alexa (Invitrogen, Burlington ON) fluorescent secondary antibodies (1:400 dilution) for 30 minutes at room temperature. TO-PRO® -3 iodide (1:1000 for 15 minutes, T-3605, Molecular Probes, Eugene Oregon) nuclear counter staining was used for confocal analyses. Citifluor® (Marivac Limited) fluorescent mounting medium was applied, and slides were coverslipped using 1-ounce, no.0 thickness cover glasses.

Confocal Microscopy

Immunocytochemically stained cultures were visualized using a Zeiss LSM 510 META confocal microscope. 1.4 oil/DIC objective lenses (plan-apochromat) ranging from 10-40X magnification were used. Pinhole diameter was maintained at 1.0-2.0 airy units for all wavelengths. Laser outputs were set at 5% (488nm), 80% (543nm) and 13% (633nm). Emission filters were used as follows: LP >633nm (Cy5), band-pass 505-

530nm (Cy2) and band pass 560-615nm (Cy3). Images were adjusted for brightness and contrast.

Table 4.1: Antibodies used in Chapter 4

Antibody	Cellular phenotype	Source and immunizing information
CRALBP	Adult Müller glia	1:1000, abcam, Cambridge MA, cat. number ab15051, monoclonal clone B2, IgG2a
GFAP	Activated Müller glia and non-retinal astrocytes	1:100, Novo Castra, Newcastle UK, cat. number NCL_GFAP_GA5, clone GA5, species and isotype mouse-anti GFAP IgG1
Glutamine Synthetase	Adult Müller glia	1:1000, Chemicon, Temecula CA, cat. number MAB302, species and isotype mouse anti-GS IgG_2. Glutamine synthetase whole protein (1-373 bp) was used as the immunizing antigen
Nestin	Neuroectodermal, stem/progenitors, RPCs and activated Müller glia	1:500, BD PharMingen, San Diego CA, cat. number 556309, clone 401, species and isotype mouse anti-nestin IgG_1
Musashi-1	Neuroectodermal, stem/progenitors and RPCs	1:1000, Chemicon, Temecula CA, cat. number AB5977, species is rabbit anti-Musashi-1
Pax6	RPCs, amacrine, horizontal and RGCs	1:500, Covance Research, Berkeley CA, cat. number PRB-278P, species is rabbit anti-Pax6
Chx10	RPCs, bipolar and a small sub-population of Müller glia	1:500, Chemicon, CA, USA, cat. numbers AB9014 & AB9016, species is sheep anti-recombinant human Chx10
Flk-1	RPCs	1:400, Chemicon, CA, USA, cat. number 05-554, species is mouse anti-KDR/Flk-1/VEGF-R2 IGg1
Notch	RPCs	1:300, Chemicon, CA, USA, cat. number MAB5352, species is mouse anti-notch presented as a GST fusion protein
Hes1	RPCs	1:1500, Chemicon, Temecula, CA, species is rabbit

Antibody	Cellular phenotype	Source and immunizing information
		anti-*Hes1*
Double cortin (DCX)	Immature, post-mitotic neurons	1:500, Chemicon, CA, USA, cat. number AB5910, species is guinea pig anti-DCX
ß-III tubulin (Tuj1)	Immature and mature neurons	1:1000, Chemicon, Temecula, CA, species is mouse anti-Tuj1
NeuN	Mature CNS neurons	1:1000, Chemicon, CA, USA, cat. number MAB377, species is mouse anti-NeuN
MAP-2	Mature CNS neurons	1:500, Chemicon, CA, USA, cat. number AB5622, species is rabbit anti-MAP-2
Hu C/D	Developing and mature CNS neurons	1:100, Chemicon, CA, USA, cat. number AB9346, species is chicken anti-Hu
Calbindin	Horizontal and RGCs	1:000, Chemicon, CA, USA, cat. number AB1778, species is rabbit anti-calbindin
Recoverin	Photoreceptors and bipolars	1:4000, Chemicon, CA, USA, cat. number AB5585, species is rabbit anti-recoverin
$p27^{kip1}$	Cell cycle exit and postmitotic Müller glia	1:1500, Chemicon, CA, USA, cat number AB3003, species is rabbit anti-p27 tumor suppression protein
CldU	Chloro-2`-deoxy-uridine	1:250, BD Accurate Chemical and Scientific Corp., Westbury NY, cat. number OBT0030, species is Rat IgG2a anti-BrdU, clone BU1/75(ICR1)

Cell Counting and Statistics

Cells were visualized on a Leica DM400 fluorescence microscope equipped with a Ludl electronic stepper stage. Positively stained cells were plotted using Neurolucida, Stereoinvestigator (Microbrightfield Systems), and all counting was performed blind to

the experimental conditions. Data were analyzed using SPSS 10 for Windows. Homogeneity of variance and normality tests were performed to ensure that the data met the requirements for parametric statistics. A One-way analysis of variance was used to evaluate main effects when comparing the number of cells present in cultures 4, 10 and 14 days post-dissection. *Scheffé post-hoc* analysis was used for multiple comparisons when main effects were significant. Data are expressed as means ± standard error of the mean (mean ± S.E.M.).

RESULTS

Müller glia Proliferate and Up-Regulate RPC Phenotypes with the Loss of Retinal Neurons In Vitro

Retinas from adult rats were dissected, dissociated and plated in high serum (10% FBS) media conditions. Two weeks following plating, the majority of retinal neurons had died, as indicated by the accumulation of cellular debris, reduction in cell number, and drastic reduction in MAP-2 and Tuj1 cell numbers (Figure 4-1A). Specifically, analysis of cell counts (mean number of cells per mm^2 +/- S.E.M., $p < 0.01$) indicated a significant decrease in the total number of Topro-3-positive nuclei present in cultures at 10 (37 +/- 6.4) and 14 days (25.2 +/- 6.8) relative to 4 days (61.8 +/- 7.4) post-dissection, indicating that retinal cell death was occurring. The reduction in cell number occurred in tandem with a significant decrease in neurons over a 2 week period, as there were significantly fewer MAP2-postive cells present at 14 days (1.6 +/- 1.1) relative to both 10 (7 +/- 4.8) and 4 (21.2 +/- 5.4) days post-dissection. Similarly, there were significantly fewer Tuj1-postive cells at 14 days (0.8 +/- 0.8) relative to both 10 (3.6 +/- 2.6) and 4 (15.8 +/- 3.4) days. In contrast, we detected significant increases in proliferation (as indicated by CldU-

positive nuclear counts) at 14 days (7 +/- 3) post-dissection when compared with both 4 (0) and 10 (0.2 +/- 0.4) days. These data indicate that proliferation within primary retinal cultures occurs concurrently with a reduction in the number of retinal neurons.

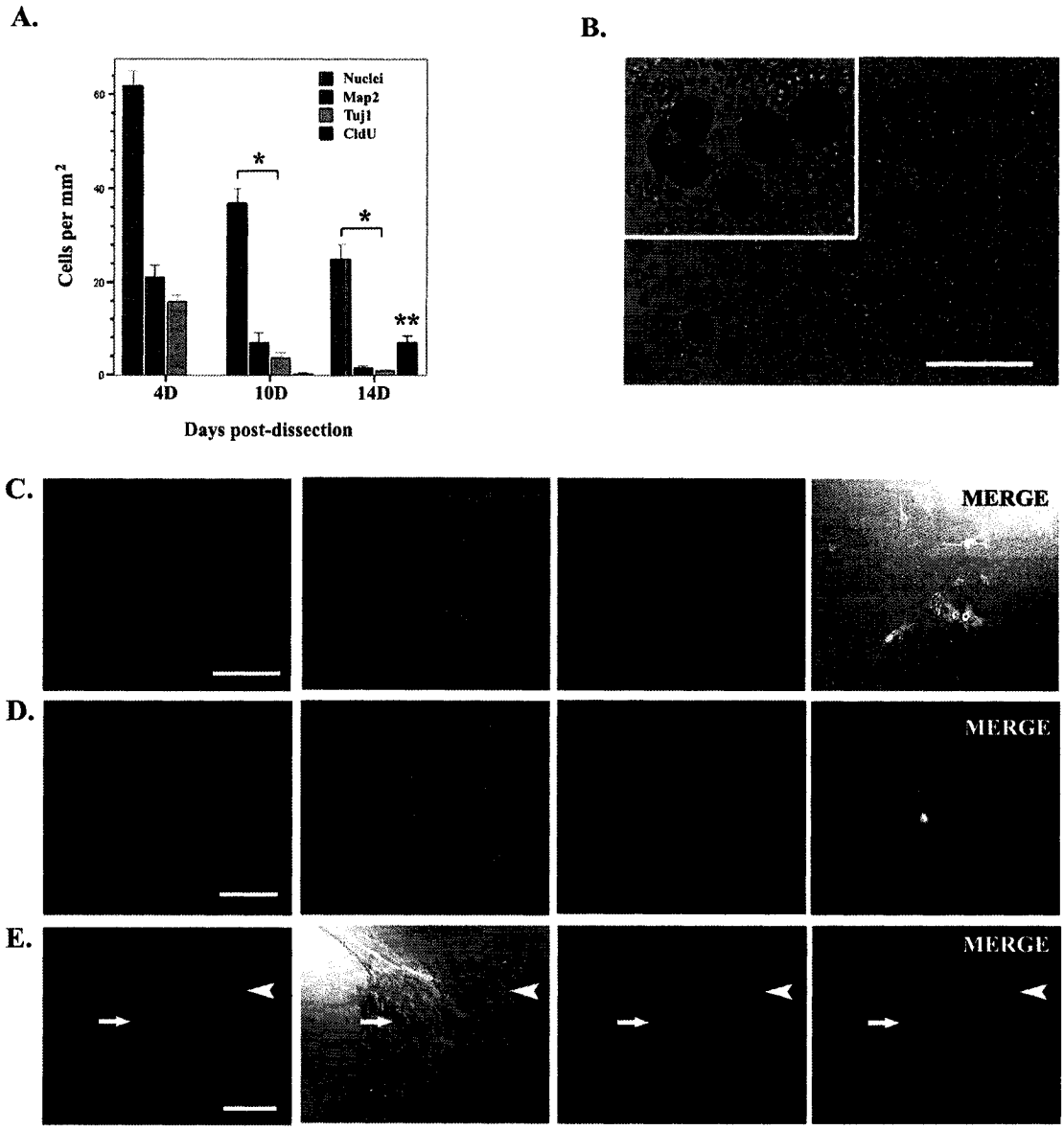
To determine whether the remaining cells present in retinal cultures 14 days post-dissection were Müller glia, we examined immunoreactivity for CRALBP. At 14 days post-dissection, a large sub-population of cells expressing CRALBP (Figure 4-1C) remained, indicating that Müller glia were present. To assess whether Müller glia were proliferating, cultures were pulsed with CldU to identify cells in S-phase of the cell cycle. Double-labeling for CldU and CRALBP indicated that Müller glia had re-entered the cell cycle, and that all dividing nuclei present were contained exclusively within CRALBP-positive cells (Figure 4-1C).

In addition to an increase in cell number, cultures maintained for 1-month contained pigmented, sphere-like aggregates that bore resemblance to previously described neurospheres derived from various regions of the developing and adult CNS (Tropepe et al., 2000; Seaberg and van der Kooy, 2003) (Figure 1b). Spheres grow to a diameter of approximately 100-200 μm, and lose pigment if maintained for greater than ~ 1-month. Unlike previously described CNS neurospheres, retinal derived spheres remain adherent to a monolayer of cells that radiate outward. Recent reports indicate that Müller glia remain in the cell cycle in the presence of reduced numbers of neurons (Close et al., 2005).

We stained CldU-pulsed cultures at 1-month post-dissection to assess whether Müller glial proliferation is correlated with the presence of retinal spheres. An absence of neuron-specific immunoreactivity (above), and the presence of CRALBP expressing cells

within spheres (Figure 4-1D), suggest that Müller glia are the principal cell type present. In addition, spheres contained centrally located, CldU-positive nuclei that co-localized with CRALBP (Figure 4-1D) suggesting that dividing Müller glia were the principal dividing cell type present, and that spheres were derived, at least in part, by Müller cell proliferation, and not cell aggregation *per se*. We assessed the expression of $p27^{kip1}$, a cyclin-kinase inhibitor which functions to mediate cell cycle exit by both RPCs (Dyer and Cepko, 2001a) and adult Müller glia (Dyer and Cepko, 2000). Staining for $p27^{kip1}$ indicated that its expression was evident within nuclei of cells migrating outward from spheres (arrowhead), and not within cells located centrally therein (Figure 4-1E) adding further evidence that spheres give rise to postmitotic progeny.

Figure 4-1. Reduced numbers of neurons in primary retinal cultures correlates with Müller cell proliferation, and formation of pigmented spheres by 1-month. (A) Primary retinal cultures were assessed for MAP2 and Tuj1 immunoreactivities. Significant reductions in nuclear (Topro-3 nuclei, red bars), MAP2 (blue bars) and Tuj1 (green bars) cell counts was seen at 10 and 14 days relative to 4 days post-plating (* = significant relative to 4 day). Conversely, a significant increase in proliferation (purple bars) was seen at 14 days compared with both 4 and 10 days (** = significant relative to 4 and 10 day). Data are mean number of cells per mm^2, $p < 0.01$. (B) By 1 month post-dissection, pigmented spheres were present, growing to a diameter of ~100-200 μm. Inset is high magnification view (C) 2-weeks following dissection, CRALBP-positive Müller glia (green) re-enter the cell-cycle as measured by CldU incorporation (red). (D) One month post-dissection, proliferating aggregates of CRALBP-positive cells resembling neurospheres are apparent, and contain centrally located, proliferating nuclei. (E) The cyclin-kinase inhibitor and negative regulator of cell cycle progression $p27^{kip1}$, is expressed in high levels in cells radiating outward from spheres (arrowhead), in marked contrast to nuclei located centrally (arrow). Scale bars = 500 μm in B, 100 μm in C and 50 μm in D-E.


A.
B.
C.
D.
E.
Nuclei
Map2
Tuj1
CldU
Cells per mm²
60
40
20
0
*
*
**
4D
10D
14D
Days post-dissection
MERGE
MERGE
MERGE

Müller glia have been shown to exhibit progenitor-like activity both in lower-vertebrate (Fischer and Reh, 2001; Wu et al., 2001) and mammalian retinas (Moshiri and Reh, 2004; Ooto et al., 2004). Our observation that spheres derived from dissected retinas were comprised of dividing Müller glia prompted us to assess whether Müller glia express phenotypes typically associated with RPCs. Cultures were maintained for 1 month, and were assessed for the expression of nestin, Musashi-1, Pax6, Chx10, Flk-1, notch and Hes1; phenotypes expressed by RPCs (Ahmad et al., 2004a; Marquardt, 2003). A sub-population of cells contained within retinal spheres express Pax6 within their nuclei (Figure 4-2A), and its expression was most frequently observed in the nuclei of cells residing in the central portions of the sphere (arrow for example). In contrast, cells residing in the periphery, and that appeared to be migrating away from the sphere, were Pax6-negative (arrowhead). Staining for the intermediate filament nestin (Figure 4-2B) revealed extensive labeling throughout retinal spheres, and in single cells residing in the surrounding culture area. Musashi-1 (Figure 4-2C), an RNA-binding protein seen in populations of neural precursors (Kaneko et al., 2000; Sakakibara et al., 1996), is expressed in both individual cells (shown) and spheres (not shown). Virtually all Müller glia express Musashi-1, as confirmed via CRALBP double-labeling (not shown). Flk-1, Chx10, notch and Hes-1 were not detected (not shown), indicating that only a select subset of progenitor phenotypes are expressed by Müller glia under these conditions. Together, these data indicate that Müller glia derived from primary retinal cultures proliferate into sphere-like aggregates, and up-regulate a select subset of phenotypes expressed by RPCs during development.

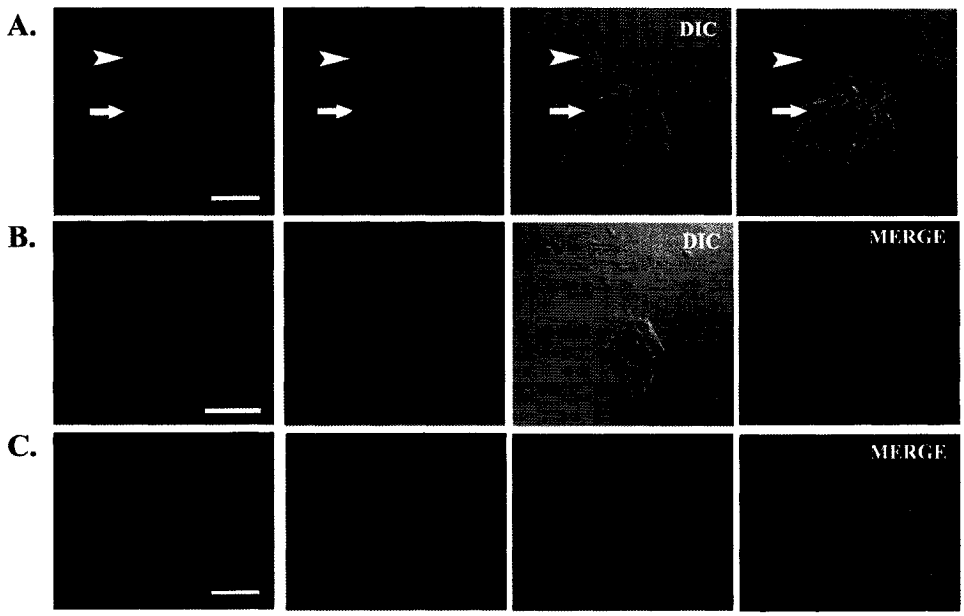


Figure 4-2. Cultured Müller glia express phenotypes akin to RPCs. (A) Centrally located cells within spheres (arrow) express the transcription factor Pax6 (green) in their nuclei (blue) whereas cells radiating outward are Pax6-negative (arrowhead). (B) Müller glial spheres and single cells express the neuroectodermal phenotype nestin (green). (C) Musashi-1-positive Müller glia (green) exclusively contain proliferating, CldU-positive nuclei (red). Topro-3-positive nuclei in all panels are blue. Scale bars = 50 μm in A and 100 μm in B-C.

Müller Glia De-Differentiate When Chronically Passaged In Vitro

Müller glial cultures were generated from C57 mouse retinas. Cultures contained a morphologically homogeneous population of cells that retain the typical bipolar appearance of Müller glia (Figure 4-3A). Immunocytochemical, RT-PCR, and electrophysiological analyses were employed to determine whether cultured mouse Müller cells exhibit characteristics previously reported in their *in vivo* counterparts. Immunocytochemical assessment indicated that all cells displayed immunoreactivity for CRALBP (Figure 4-3A). In addition, RT-PCR analysis of total RNA derived from cultures demonstrated the expression of CRALBP transcripts (Figure 4-3B), substantiating immunolabeling results. One physiological characteristic that distinguishes glial cells from neurons is their high conductance to K^+, that itself is sensitive to micromolar concentrations of extracellular $Ba2^+$. Electrophysiological analysis using patch-clamp recordings demonstrated that cultured mouse Müller cells exhibit a large inward and outward conductance (Figure 4-4C). Furthermore, perfusion with media containing 100μM $Ba2^+$ was able to decrease the amplitudes of all K^+ currents, at hyperpolarizing potentials (Figures 4-4 A & B). Together, these data indicate that cultured Müller glia retain basic phenotypic, genomic, and electrophysiological characteristics present in their *in vivo* counterparts.

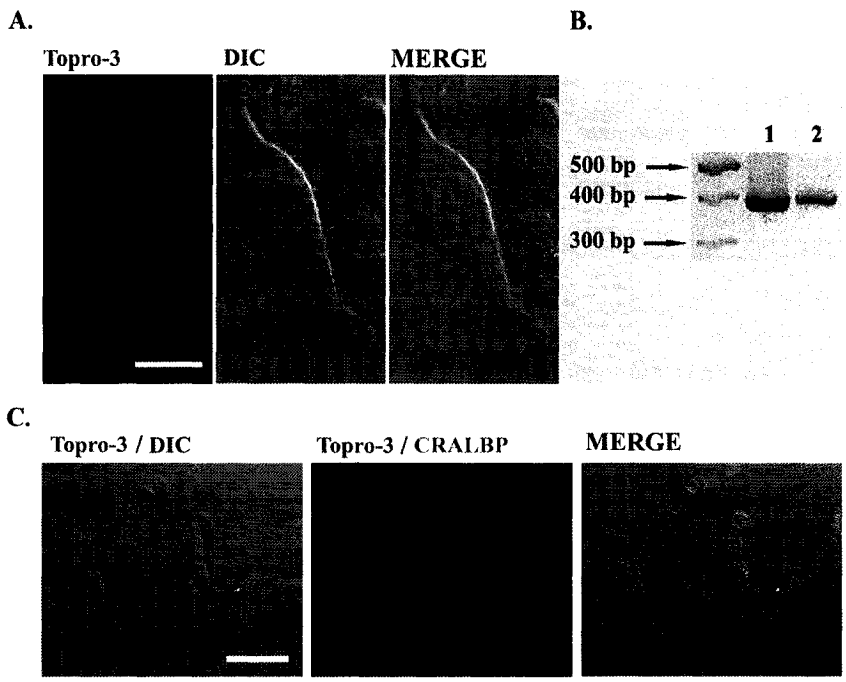


Figure 4-3. Purified mouse retinal cultures contain a population of cells that exhibit phenotypic and genotypic characteristics of Müller cells. (A) Cells retain the basic bipolar morphology typical of Müller glia. (B) RT-PCR analysis confirmed the presence of CRALBP transcripts in total RNA derived from Müller cell cultures. Both retina (lane marked 1) and cultures (lane marked 2) show bands at 400 bp. (C) Ubiquitous CRALBP immunoreactivity (RED) confirms its expression in all cultured Müller cells. Topro-3-positive nuclei are BLUE. Scale bars = 20 μm in A and 50 μm in C.

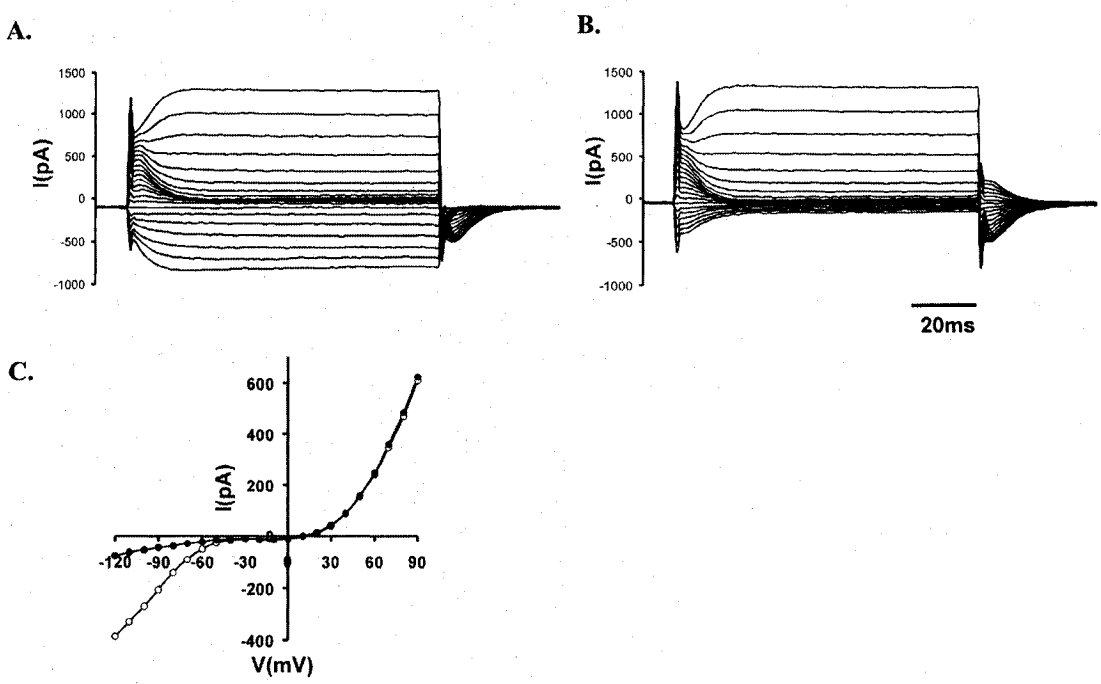
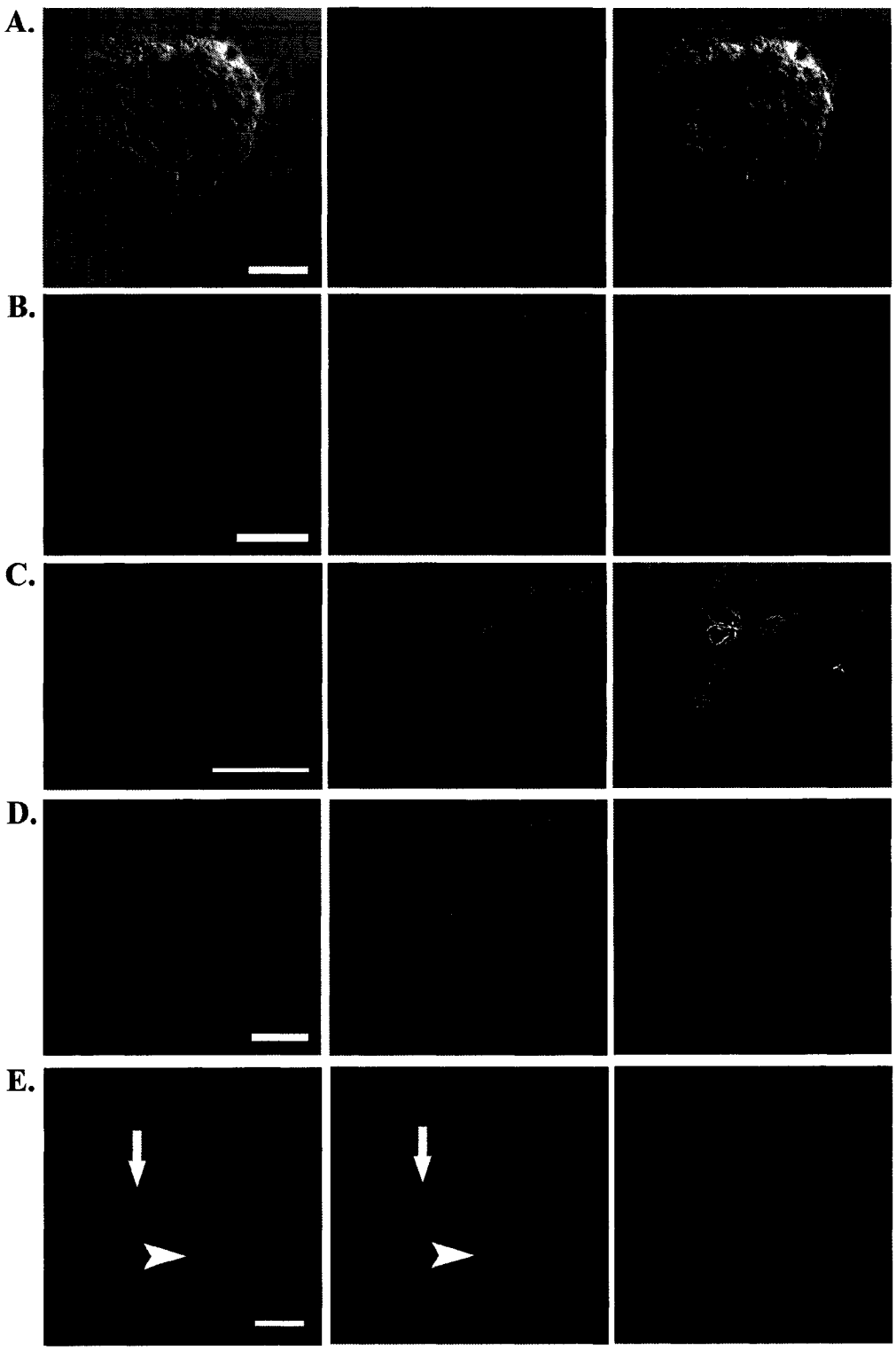


Figure 4-4. Cultured mouse cells display cardinal electrophysiological features of Müller glia, as determined by voltage-clamp recordings and their sensitivity to $BaCl_2$. (A) Whole-cell currents in Müller cells were elicited with voltage steps between -120 and +90 mV, from a holding potential of -60 mV, and in 10 mV increments. (B) The inward current was reduced in the presence of 100 μM $BaCl_2$. Current/voltage (I-V) relation for currents obtained from Müller cells as shown in A and B in the absence (open circles) and presence (closed circles) of 100 μM $BaCl_2$. (C, $\underline{n} = 8$).

Cells in these cultures undergo a very high rate of proliferation as an adherent monolayer, and can be maintained by passaging under high serum (10%) conditions. To assess whether chronically passaged Müller cell cultures retain phenotypes reminiscent of either Müller glia, and/or stem/progenitor cells, we assessed cultures that had been passaged greater than 25 times. Immunocytochemical analysis indicated an absence of immunoreactivity for glutamine synthetase, indicating a downregulation of a phenotype typically associated with Müller cell identity (not shown). After observing that these cells were not immunopositive for GS, we sought to determine whether the reduction in this phenotype was co-incident with the up-regulation of a selection of phenotypes associated with RPCs. Chronically passaged cells expressed high levels of the VEGF receptor, FLK-1, predominantly in cells located in the central portions of spheres (Figure 4-5A) suggesting an emerging, RPC-like identity (Yang and Cepko, 1996). In addition, cytoplasmic labeling for the neuroectodermal, intermediate filament nestin (Figure 4-5B) both in single cells and spheres was evident. In contrast to primary cultures assessed at 1-month post-dissection (above), the expression Pax6 was not detected in chronically passaged cultures (not shown). Assessment of the transcription factor Chx10, however, revealed that a sub-population of chronically passaged cells express Chx10 (Figure 4-5C), indicating that RPC-like transcriptional mechanisms are present. Consistent with a recent report that Müller cell, progenitor-like activity is also co-incident with the expression of the early and late RPC phenotype, notch-1 (Das et al., 2006), we observed notch-1 expression in single cells (Figure 4-5D) and spheres. To determine whether cultured Müller glia up-regulate notch related effectors, we assayed for the positively regulating, down-stream transcription factor for notch, Hes1 (Figure 4-5E). A large

proportion of cells contained punctate Hes1 immunoreactivity in the cytoplasm (arrow), and/or nuclei (arrowhead), indicating that notch signaling may be active. Together, these data demonstrate a shifting pattern of phenotypes expressed by chronically maintained, cultured Müller glia. This shift is suggestive of a process which resembles de-differentiation, as indicated by the downregulation of glial phenotypes and the up-regulation of neural and retinal stem/progenitor cell phenotypes.

Figure 4-5. Chronically maintained Müller glial cultures express phenotypes seen in early, multipotent RPCs. (A) Spheres generated by proliferating Müller glia express the VEGF receptor Flk-1 (green). (B) Both single cells and spheres (not shown) express nestin (green), (C) Chx10 (green), (D) notch (green) and (E) the notch signaling transcriptional regulator, Hes1 (red). Hes1 immunoreactivity is localized to punctuate staining in the cytoplasm (arrow) and the nucleus (arrowhead). Scale bars in A-D = 50 μm and 20 μm in E.


A.
B.
C.
D.
E.

Growth Factor Exposure Induces the Expression of Neuronal Phenotypes by Cultured Müller Glia In Vitro

Chronically maintained Müller glial cultures were assessed for the presence of cells with morphological and immunocytochemical resemblance to developing (DCX and βIII-tubulin) and adult (NeuN, MAP-2 and βIII-tubulin) neurons, as well as for retinal cell-specific (Calbindin D-28k for horizontal cells, Pax6 for amacrine cells, Chx10 and PKCα for bipolar cells, and recoverin for photoreceptors) phenotypes. Under conditions used for colony expansion (serum containing media), none of the cells present in cultures displayed any morphological or immunocytochemical phenotypes of neurons (not shown). To determine whether Müller glial cultures, when exposed to differentiating conditions conducive to neuron production are capable of up-regulating adult neuronal phenotypes, we exposed cells to media containing low-serum, and low-serum accompanied by EPO, BDNF, or EPO/BDNF in combination. Cultures exposed to low-serum differentiation conditions for 7-days contained a small number of cells that bore morphological resemblance to neurons (not shown). Staining revealed that a small population of cells were immunpositive for DCX (Figure 4-6A) or βIII-tubulin (Tuj-1) (Figure 4-6B). The morphology of immunopositive cells, however, was not suggestive of neuronal differentiation. In support of this, staining for mature neuronal phenotypes (HuC/D, Calbindin, recoverin, NeuN) revealed an absence of expression within low-serum cultures, indicating an impairment of these cells to fully differentiate.

Following treatment of cultures with growth factors, however, the emergence of cells with morphological features of neurons was evident as early as 24 hours after treatment (Figure 4-6F). Although treatment of cultures with BDNF or EPO alone did not elicit a change in phenotype, following 7-days of exposure to EPO and BDNF in

combination generated a sub-population of Müller glia that up-regulate Hu C/D (Figure 4-6C), Calbindin (Figure 4-6D), or recoverin (Figure 4-6E). Interestingly, Hu C/D expression elicited by growth factor treatment was contained almost exclusively within cells progressing through M-phase, as indicated by condensed chromatin aligned at the metaphase plate (Figure 4-6C). Cell counts (Figure 4-6E) indicated that only a small percentage (mean % of total nuclei +/- S.E.M.) of cells express DCX (5.7 +/- 1.7), Tuj1 (10.5 +/- 2.4), HuC/D (10.4 +/- 1.9), calbindin (7.3 +/- 3) or recoverin (12 +/- 2.9). It is clear that growth factor regimen used was not sufficient to induce the production of cells resembling all classes of retinal neurons, as indicated by the absence of Pax6 (amacrine cells), Chx10 or PKC alpha (bipolar cells) expression within cells displaying neuronal morphology. These data indicate that when exposed to differentiation media, including that which contains growth factors, Müller glia can generate some progeny with immunocytochemical and morphological features of developing and adult neurons.

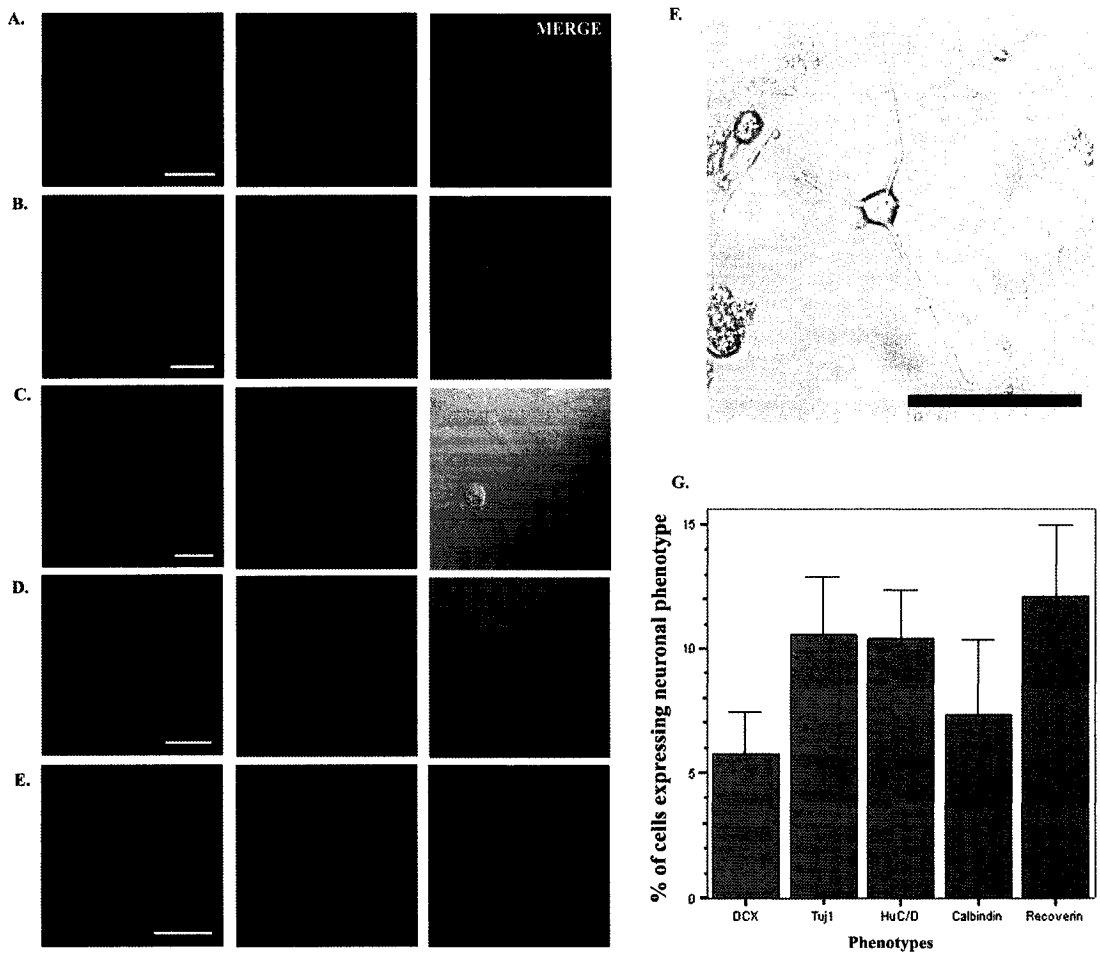


Figure 4-6. Chronically passaged Müller glia can be stimulated to express phenotypes reminiscent of developing and adult retinal neurons. Reduction in serum levels results in the expression of (A) DCX, (B) Tuj1 – β-III tubulin in cells which do not resemble neurons. Following 7 days of growth factor exposure, cultures contained cells which express (C) Hu C/D (D) calbindin D-28k or (E) recoverin. Although not immunoreactive for adult neuronal markers, cells exhibiting a neuronal morphology (F) are evident as early as 2 days following growth factor treatment. (G) Approximately 5-12 % of cells present in cultures express at least one of the neuronal phenotypes assayed following 7 days of growth factor exposure. Scale bars in A,B and E = 100 μm, C, D and F = 50 μm.

Discussion

Müller glia are quickly emerging as candidates for therapeutic-based, cell replacement in the adult retina. It is evident from previous work, and those results presented here, that Müller glia retain many cardinal phenotypic and functional features of RPCs. In this report, we demonstrate that adult mammalian Müller cells proliferate, and up-regulate phenotypes akin to neural stem/progenitor cells (nestin and Musashi-1) and RPCs (Pax6) when cultured in the presence of stem cell expansion media conditions. In addition, proliferating Müller glia generate sphere-like aggregates comprised of cells that express a select subset of RPC phenotypes. When chronically maintained in culture over many passages (>25), Müller glia undergo a process that resembles de-differentiation including the down-regulation of GS, and an up-regulation of Chx10, notch, Hes1 and Flk-1. Finally, when exposed to differentiation conditions including that which contains growth factors, chronically passaged Müller cells are capable of generating progeny that bear phenotypic resemblance to developing (DCX & ß-III tubulin) and adult (Hu C/D, calbindin, recoverin and ß-III tubulin) neuronal progeny.

Proliferation of Cultured Müller Glia is Associated With the Loss of Retinal Neurons

By postnatal day 10.5 in rodents, the ocular environment has shifted from that which encourages the proliferation of remaining neuroepithelial RPCs, to one that induces their exit from the cell cycle. A number of secreted factors (growth factors and biosynthetic precursors) and cell adhesion molecules (delta/notch signaling) have been implicated in governing aspects of cell cycle dynamics and fate determination within the retina (for review see Dyer and Cepko, 2001b). The proliferative activity of adult Müller glia has been stimulated by injury to the retina (Fischer and Reh, 2001; Ooto et al., 2004),

and by exposure to mitogenic growth factors both *in vivo* and *in vitro* (Das et al., 2006). Under similar dissection and culture techniques to those used in our experiment, a previous study used fibroblast growth factor-2 and EGF to establish Müller cell spheres (Das et al., 2006). In contrast, we describe the re-entry of Müller glia into the cell cycle in the absence of exogenously applied growth factors. Although chronic passaging can result in cell transformation, and changes in gene expression, we speculate that either the removal of neuron-derived cues, or factors released as a function of neuronal death are more likely to stimulate Müller cell proliferation. The 2-week delay in Müller cell proliferation post-dissection contrasts the response of Müller glia following neurotoxic injury in chick, however, wherein the proliferative response is initiated at 2 days post-lesion (Fischer and Reh, 2001). Although the culture environment differs considerably from that seen *in vivo*, many congruent aspects of the pathophysiology of retinal cell death are retained *in vitro* (Levin, 2005). Differences in the rate and extent of cell death seen following excitotoxic lesioning and/or species differences may be responsible for these differences.

One recent report attributes an enrichment of neurogenic potential by Müller glia following excitotoxic lesioning *in vivo*, in part, by activation of notch and wnt signaling (Das et al., 2006). Although it is unclear whether injury induces notch signaling in Müller glia, notch up-regulation is coincident with other RPC phenotypes in our cultures. Our results indicate that the expression of notch and Hes1 is not evident during the early culture phase, and is detected only after multiple passages. These data suggest that notch signaling may not necessarily correlate with injury, but rather the process of de-differentiation itself as a consequence of one or multiple etiologies.

Neurogenic Competence and Responsiveness to Growth Factor Exposure are Retained by Adult Mammalian Müller Glia

One generally accepted framework of RPC function proposes that progenitors undergo changes in competence states, and that those changes may be influenced by signaling initiated by epigenetic factors (Cepko, 1999). Congruent with this, CNS neural precursors from adult brain can be directed to generate specific classes of cells in response to appropriate growth factors both *in vitro* and *in vivo* (for reviews see Hagg, 2005; Seaberg and van der Kooy, 2003). We explored the response of cultured Müller glia to two growth factors, EPO and BDNF, each of which has been shown to increase the production of neurons from populations of NPCs in brain (Shingo et al., 2001; Zigova et al., 1998). Although the yield of neuron-like cells generated by Müller cultures was low (5-12%), growth factor exposure did stimulate the production of cells with phenotypes (Hu C/D, Calbindin & recoverin) expressed in retinal neurons (Figure 4-6). In brain, EPO treatment increases neuron production by subventricular zone NPCs, an effect that was coincident with the expression of the bHLH transcription factor Mash1 (Shingo et al., 2001). Similar transcriptional mechanisms govern the production of distinct cell types within the retina during development, wherein Mash1 expression has been shown to increase the propensity toward rod, cone and bipolar cell production at the expense of Müller cells (Tomita et al., 1996). Based on this, we hypothesized that the production of rod-like (recoverin-positive) cells following EPO/BDNF exposure may be co-incident with Mash-1 expression. Although we assayed for the expression of Mash1 during the expansion and growth factor-mediated differentiation of Müller cell cultures, only low-level immunoreactivity was detected in all conditions (not shown) suggesting that other intrinsic signaling mechanisms may be involved. We also describe an impaired capacity

for many neuron-like cells produced following growth factor treatment to fully differentiate, an observation that is typical of cells produced by Müller glia in other vertebrate species (Fischer et al., 2004; for review, see Fischer and Reh, 2003b). Inappropriate trophic support, insufficient exposure to other differentiating factors, and the lack of functional connectivity are all factors that may contribute to this observation.

Conclusions

Müller glia are gaining momentum as candidates for cell replacement therapy. The recent discovery of multipotentiality and self-renewal by cultured Müller glia provide proof of principal evidence that the production of distinct cell types may be possible. Our data indicate that Müller glial proliferation correlates with the reduction in retinal neurons when cultured, and that their treatment with specific growth factors is effective in producing cells expressing neuronal phenotypes, an effect that is co-incident with the up-regulation of appropriate transcriptional regulators. Understanding the responsiveness of Müller glia to exogenous, lineage restricting growth factors such as those described here, will be necessary for the development of effective cell replacement therapy.

CHAPTER 5: Discussion

Summary of Thesis

The experimental results in this thesis yield three main findings: (1) selective injury to RGCs elicits a progenitor-like response by cells of the CB, including re-entry into the cell cycle, and up-regulation of RPC and adult retinal phenotypes; (2) treatment of adult mammalian eyes with EGF and EPO stimulates the proliferation and interkinetic nuclear migration of Müller glia, as well as Müller cell expression of RPC phenotypes; and (3) isolation of Müller cells and exposure to stem cell-promoting conditions *in vitro* stimulate their de-differentiation into a cell that resembles a RPC, and further exposure to growth factors induces their up-regulation of neuronal phenotypes.

The goal of this dissertation was to activate endogenous neural precursors in the adult mammalian retina via exposure to selective injury within the retina, or the exogenous application of growth factors. Historically speaking, it has been understood that the adult mammalian retina exhibits no cell replacement activity, which has led to the long held belief that it was subsequently devoid of RPCs post-developmentally. Research by Tropepe and colleagues (2000), however, identified the existence of a population of quiescent, *bonafide* adult mammalian retinal stem cells, thus rekindling interest in adult retinal neurogenesis. Other well characterized populations of adult neural stem/progenitors have been shown to respond to injury, as well as exposure to compounds that exert mitogenic and morphogenic influences (for review, see Hagg, 2005). Thus, we sought to determine whether similar progenitor-like responses were inducible in precursors of the adult mammalian eye. Using well characterized methods of retinal ganglion cell (RGC) injury, intravitreal injections of growth factors, as well as cell

culture techniques, we assayed for hallmark progenitor-like responses by cells which reside in the adult rodent eye.

By their very nature, active progenitor cells display a number of keystone phenotypes that permit their identification. These phenotypes can involve the re-entry into the cell cycle or up-regulation of regulatory elements required for the maintenance of progenitor potential. Functional progenitor cells must, however, generate progeny that can successfully migrate to appropriate stereotypical positions within the retina, differentiate, and re-integrate or re-establish functional circuitry. My *in vivo* research provides: **(1)** a link between RGC injury and activation of adult retinal progenitors (Chapter 2); **(2)** a strategy for Müller cell activation via the use of EGF; and **(3)** a strategy (with the addition of EPO) for manipulating the expression of RPC phenotypes in activated Müller glia, that suppresses the differentiation of newly generated cells into glia and alters the distribution of those progeny throughout the retinal layers (Chapter 3). My *in vitro* data (Chapter 4) support findings from Chapter 2 pertaining to the regulation of progenitor-like behavior by retinal neurons, and also support my findings from Chapter 3 by demonstrating that the Müller cell possesses neurogenic-like growth factor responsiveness. In addition, results presented in Chapter 4 show that when cultured Müller glia are maintained in the absence of neurons for a sufficient period of time, they display a more robust panel of progenitor features, including up-regulation of notch, Flk-1, and formation of spheres. These results, based on the various profiles of phenotypes expressed in response to differing conditions, suggest that Müller glia can reside at various positions on the continuum of stemness. Specifically, *in vivo* activation by EGF elicits the expression of nestin and Pax6 only (Figures 3-5 and 3-6), and does not result in

neuron production, indicating a limited precursor-like response by these cells. *In vitro*, however, Müller glia can be induced to up-regulate a number of RPC phenotypes both in acute culture times (Figure 4-2; Pax6, nestin, musashi-1) and progressively more robust and primordial RPC phenotypes when chronically maintained (Figure 4-5; notch, Hes1, Flk-1, Chx10, nestin). This occupation of multiple states on the "stemness" continuum, at least with respect to the profile of phenotypes expressed, demonstrates the considerable phenotypic plasticity of these cells, and suggests that adult Müller glia retain some capacity for de-differentiation.

My research also reveals some limitations of Müller glia with respect to functional cell replacement. The differentiation and re-integration of cells produced as a function of *in vivo* manipulations is not evident in this thesis. Newly generated cells in response to EGF exposure do migrate to various layers of the retina, but fail to differentiate into neurons. This failure to differentiate into neurons, rather than being accompanied by the default differentiation into glia, is co-incident with a large proportion of cells arrested in an uncommitted state that is neither glial nor neuronal. *In vitro*, I was unable to stimulate the production of phenotypic expression of all retinal cell types, and was also unable to generate significant numbers of neuron-like progeny.

In light of my discoveries, with respect to both the promising characterization of progenitor behavior by Müller glia, and the considerable limitations of that behavior therein, I will discuss: **(1)** mechanisms that may be responsible for this RPC-like response, including that of retinal damage and/or factors released by RGCs; **(2)** the limitation of that response relating to a limited complement of RPC gene expression; and **(3)** strategies that may be used to push further the response of these cells toward

functional cell replacement. In addition, based on the data presented in Chapters 2-4, I speculate on **(4)** how we might define Müller glia in the context of existing frameworks of adult NPCs. **(5)** Finally, I discuss whether adult Müller glia are sufficient substrates for neural repair in the adult retina. Sections of this Chapter have been submitted for publication.

Activating Adult Retinal Precursors: Retinal Damage vs. RGC-Derived Cues

Damage, Reactive Gliosis and Progenitor Response by Adult Precursors

In this section, I discuss the contrasting mechanisms of adult retinal precursor cell activation, with emphasis on injury vs. growth factor exposures. The expression of keystone features of reactive gliosis within populations of adult NPCs hampens the observer's ability to specifically identify these cells with certainty. A subset of these characteristics found in reactive astrocytes, including cell cycle re-entry and up-regulation of intermediate filaments is congruent with the functioning of many NPCs in brain. Intermediate filament expression mediates changes in cellular ultrastructure during the processes of cell division (including cytokinesis) and process extension. From a biological perspective, it is understandable that common molecular mechanisms may be used to mediate very different biological events, as in a cell reacting to injury or dividing to generate a neuron. However, subsets of these intermediate filaments have been used to phenotypically screen for NPCs and RPCs, including nestin (or transitin in the chick), and vimentin.

In Chapters 2-4, I describe the up-regulation of various subsets of transcriptional and filamentous phenotypes normally associated with RPCs, within adult ocular cells.

Interestingly, these results vary as a function of the conditions surrounding the modality of cell activation, indicating a very stimulus-specific pattern of expression. For example, nestin and GFAP are both up-regulated following EGF-treatment (Chapter 3, Figure 3-5), but neither is present in retinas that undergo selective RGC death following axotomy (Chapter 2), an observation that contrasts previous findings (Xue et al., 2006b). The up-regulation of intermediate filaments, being a classic feature of reactive gliosis both in brain astrocytes and in Müller glia (for review see Pekny and Pekna, 2004), would lead us to predict their expression following axotomy. In fact, the discovery of neurogenic Müller glia in chick was established by stimulating them using excitotoxic retinal injury (Fischer and Reh, 2001; Fischer and Reh, 2003b), and was then followed by the discovery that they can also be activated using exogenous growth factor treatments (Fischer et al., 2002b). It is clear from Fisher's work, however, that excitotoxic lesioning results in an aggressive cascade of cell death within a short time (approximately 2-days). Optic nerve axotomy used in our study, however, results in a delayed onset of RGC apoptosis followed by cell death which proceeds over a 10-day period (Berkelaar et al., 1994). This contrast in the temporal progression in RGC death may explain differences in Müller cell response. Müller glia respond to retinal pathology by buffering neurotransmitter concentrations and maintaining homeostasis within the tissue (for review, see Bringmann et al., 2006). During acute excitotoxic damage, it is possible that neurogenic Müller glia are overwhelmed by the highly acute release of factors by large numbers of RGCs, and respond via similar mechanisms to those that are up-regulated during reactive gliosis.

The congruencies between Müller glial neurogenic response and reactive astrocytes, including the up-regulation of intermediate filament proteins and re-entry into the cell cycle, may underscore the crosstalk between gliotic mechanisms and those that signal de-differentiation into a progenitor state. Based on this crosstalk, mechanisms active in gliotic Müller cells may stimulate a sub-population of Müller glia to re-activate RPC genes, and become neurogenic. This damage-induced response has been previously described in the adult cortex, and resulted in the discovery of a novel population of NPCs. Specifically, induction of apoptosis in neurons residing adjacent to GFAP-expressing, neurogenic astrocytes results in their re-entry into the cell cycle, and production of viable neuronal progeny (Magavi et al., 2000). Surrounding astrocytes exhibited a typical reactive gliotic response, demonstrating that parallel mechanisms of progenitor activity and gliosis stimulate contrasting cellular behaviors, possibly within a heterogeneous population of GFAP-expressing cells.

The magnitude of pathology appears to contribute to whether cells exhibit mild reactive gliosis, or profound re-entry into the cell cycle and neurogenic activity. In the retina, RGC death following ON axotomy has been shown to induce mild Müller cell reactivity , including up-regulation of nestin (Xue et al., 2006a; Xue et al., 2006b), although this effect is not sufficient to induce re-entry into the cell cycle. Excitotoxic lesions using agents that are less selective (i.e. NMDA) have recently been shown to induce Müller cell proliferation in the mammal as well (Ooto et al., 2004; Dyer and Cepko, 2000), supporting the idea that profound and acute damage is required for their activation. Based on these observations, it appears as though Müller glia respond to

graded damage within the retina, supporting the idea that a neurogenic response by Müller glia can be partially regulated by gliotic mechanisms.

In chick, the selective response to damage is even more evident when excitotoxic ablation of single neuron subtypes is performed. Colchicine selectively destroys RGCs in the avian eye, and elicits a similar neurogenic effect to that seen using NMDA, which is not selective to RGCs. What is interesting, however, is that colchicine induces the genesis of Brn3b-positive progeny, indicating that RGCs are being replaced following their selective loss (Fischer and Reh, 2002).

Our data in the adult rodent indicate that exposure to high levels of mitogens is sufficient to induce an aggressive proliferative response by Müller glia (Figure 3-3). We also demonstrate, however, that multiple etiologies are able to stimulate Müller glial re-entry into the cell cycle. During the initial culturing of dissected retina, Müller glial proliferation is delayed until an almost complete loss in neurons, suggesting that inhibitory cues, rather than cell death, may be responsible for Müller cell quiescence (discussed below).

Retinal Ganglion Cells as Regulators of Adult Retinal Progenitor Behavior

In Chapter 2, we demonstrate that the selective loss of RGCs in the adult murine retina results in both a proliferative response (as measured using BrdU histochemistry) and transcriptional up-regulation akin to that seen in active RPCs during development, within CB cells. The anatomical location of these CB cells corresponds with the location of previously described adult retinal stem/progenitor cells. In addition to progenitor-like activity, cells within the CB respond to RGC damage by up-regulating an immunological feature (recoverin) of photoreceptors and/or bipolar cells, an effect that is independent of

proliferation. These observations provide evidence that the activity of quiescent retinal precursors in the adult eye is linked to signaling provided by either RGCs, or factors released as a function of RGC injury. We also demonstrate, however, that the presence of neurons influences the quiescence of adult, precursor-like Müller glia. The re-entry of Müller glia into the cell cycle (Figure 4-2) and their up-regulation of RPC phenotypes (Figure 4-3) are negatively correlated with the presence of neurons in culture.

So, do RGCs (or retinal neurons in general) regulate quiescence in the adult retina? Proof of concept experiments indicate that basic inhibitory feedback mechanisms do exist in the development of the retina (Gonzalez-Hoyuela et al., 2001). There is significant anatomical segregation of RGCs and CE progenitors, suggesting that diffusible factors are responsible for the induction of progenitor-like activity in the CB following RGC injury. Historically, it was clear that the gross ocular patterning seen during early eye development, as well as the activity of RPCs, was under partial regulation by diffusible, non-cell autonomous signals (for review, see Chow and Lang, 2001). The sources of these signals were identified as being adjacent structures such as the surface ectoderm and paraocular mesenchyme. Similar influences exerted by diffusible factors within the retina have been shown to govern the production of individual retinal cell types during retinogenesis. This was demonstrated with the discovery that NGF, released from newly generated RGCs, inhibits the further production of RGCs (Gonzalez-Hoyuela et al., 2001). This autoregulation maintains the correct number of RGCs produced by RPCs, such that a sufficient number of RPCs are later available to generate the required numbers of other classes of retinal neurons. It has since been shown that the regulation of RPC proliferation is also influenced by RGCs in both

early and later periods of retinal development (Mu et al., 2005). In these experiments, RGCs were selectively ablated using Cre recombinase control of diphtheria toxin expression, which was selectively expressed in RGCs expressing both Brn3b and six3. The loss in RGCs, and in turn the autoregulatory influence on RPCs, resulted in a reduction in RPC proliferation. These findings coincide with the previously discovered role of RGC-derived shh in retinal patterning (Wang et al., 2005). These elegant studies provided firm evidence that progenitor function was under partial control by as few as a single class of retinal neurons. In lower-vertebrate retinas, Fisher and colleagues demonstrate that the activity of ciliary marginal zone progenitors is under partial regulation by a single subtype of amacrine cell containing glucagon (Fischer et al., 2005b). Data derived from this series of studies clearly show that progenitor function can be influenced by positive and negative regulators derived from not only classes, but also sub-classes, of retinal cells.

Limitation of Progenitor Response and Strategies for Cell Replacement

Undifferentiated Cells May Not Be Such a Bad Thing

When observing the response of a population of NPCs to exogenous activation, there is often considerable degree of heterogeneity in the cellular responses of individual cell subtypes within that population. This heterogeneity may include a failure of a cell to differentiate into any class of cell, or a limited production of a single cell type. In this thesis, I describe both of these observations, wherein EGF-induced proliferation generates cells that do not label for differentiated neurons or glia, or that growth factor treatment *in vitro* is only effective in generating progeny that resemble two classes of

retinal neurons (photoreceptors and RGCs; Figure 4-6). Early observations by Fischer and colleagues described a residual population of undifferentiated, uncommitted, and recently-divided cells that linger around following excitotoxic injury in the chick retina. These cells, unlike their neurogenic neighbors, were unable to progress into a fully differentiated neuron or glial cell. Although the failed differentiation of these cells can legitimately be regarded as a shortcoming in our efforts, we can state that these cells may serve as substrates that are primed and ready for forced differentiation into neurons.

Developing strategies aimed at pushing undifferentiated cells towards becoming neurons requires us to understand what genes are activated with various exogenous factors, as well as understanding the cumulative effects of multiple factors in a treatment cocktail. In Chapter 3, it became evident that when used in combination, EGF and EPO act both synergistically and antagonistically with one another. Specifically, EGF exposure resulted in a small proportion of Müller glia expressing Pax6, whereas EPO treatment elicits its robust up-regulation (Figure 3-6). These data are the first to demonstrate that these particular growth factors are able to up-regulate Pax6 in this cell type. It is also evident, however, that combinatorial use of these factors can prove to be antagonistic with respect to transcriptional activation. For example, we describe a potent ability of EPO in up-regulating Pax6 in Müller glia, but a significant reduction of that effect when accompanied by EGF and EPO in combination (Figure 3-6). These results indicate that the robust Pax6-promoting affect of EPO can be overridden by co-treatment with EGF, and underscores the antagonistic affects that these two compounds can exert. In contrast, synergy between EGF and EPO is also evident. EGF and EPO-treatments

independently are not sufficient to up-regulate Chx10 in Müller glia, whereas their co-treatment is able to induce this response (Figure 3-7).

To further complicate this delicate interaction playing out in the context of adult retinal tissue, salient inhibitory cues associated with the presence of adult retinal neurons (Figure 4-1), and a latency of Müller cells to up-regulate primordial RPC phenotypes until having undergone multiple rounds of mitosis, may both impede our goal of generating neurons. These observations underscore the multiple parallel mechanisms involved in RPC function, and the multiple etiologies for their activation and inhibition. When we consider that multiple growth factors, and the influence of existing retinal tissue, are all interacting in a dynamic milieu, the prospect of directing the neurogenic activity of Müller glia with any degree of fidelity becomes an ominous goal. For these reasons, a "dirty" approach which exposes these cells to a large cocktail of growth factors, each aimed at activating one or more of the many genes required for successful neurogenesis, will likely not be successful.

Early studies suggest that master regulators of organogenesis, such as Pax6 in the case of eye development, lie upstream of many genes required for eye formation, and were regarded as glimmers of hope for stimulation of multipotentcy. These master genes were hoped to be sufficient to initiate a cascade of RPC gene expression that would be sufficient for the production of all retinal neurons. Unfortunately, the potency of these genes, including that of Pax6, to do this was only observed in the developing organism. Although the ectopic expression of Pax6 stimulates the formation of a rudimentary eye in lower animals (Halder et al., 1995; reviewed by Chow and Lang, 2001), it is evident that Pax6 expression in any cell in the mature retina is not, in itself, sufficient to stimulate the

production of neurons, as it is present in postmitotic amacrine cells, and its expression in Müller glia is not sufficient to induce neurogenesis. That being said, one shortcoming of neurogenic research in the adult is that the recapitulation of major regulating mechanisms involved in retinal development in adult animals does not necessarily translate to the same cytogenic response seen developmentally. This decreased ability in the adult may, in large part, be due to the tissue context and the shift in competence associated with NPC quiescence.

Defining Müller Glia in a Framework of Stemness

Adult Retinal Precursors Do Not Closely Resemble the In Vivo Functional Potency of Their Developmental Counterparts

Quiescent neural precursors respond to activation by de-differentiating, re-entering the cell cycle, up-regulating progenitor gene expression, and generating progeny via either symmetrical or asymmetrical division. The process of de-differentiation includes the up-regulation of cell cycle machinery, including those genes belonging to the cyclin and cyclin dependent kinases (reviewed in Dyer and Cepko, 2001b). Cell cycle re-entry is under strict control of transcription factors whose function lie upstream of cyclin activation. In the retina, those factors include Pax6 and Chx10 (reviewed in Fischer and Reh, 2003b), genes that are up-regulated in active RPCs during phases of proliferation and differentiation. During the process of cell proliferation, non-cell cycle-related proteins that correlate with progenitor cell behavior are also up-regulated. We and others have established, however, that adult retinal precursors do not up-regulate all factors that have been shown to be expressed during primordial eye development. Early markers such as Rx1, Isl-1, Crx and Six3, as examples, have not been described in active adult retinal

precursors. These genes are expressed in cells ranging from optic primordia to precursors destined to become postmitotic photoreceptors (reviewed by, Marquardt, 2003). The restricted progenitor activity in adult Müller glia likely reflects this truncated regimen of gene expression.

The restricted progenitor potential of adult Müller glia compels us to make a decision regarding where on the continuum of stemness these cells lie. Based purely on these phenotypical criteria, one might propose that Müller glia do not deserve the prestigious title of retinal stem cell, and are likely only capable of acting as restricted progenitors. This statement, however, ignores the historical trend in the characterization of stemness within populations of adult NPCs. Early experiments describing the gliotic response to injury in the brain had focused on the classic gliotic response, and the roles of reactive glia were relegated simply to a response to damage as opposed to cell replacement. The line between reactive gliosis and RPC-like activity, however, has been increasingly blurred in the last decade, with the discovery that many congruent mechanisms are evident within both phenomena. In fact, the neural response to specific modalities of damage almost always results in activation of resident glia (i.e. GFAP up-regulation in astrocytes in brain), including re-entry into the cell cycle. In areas of the CNS that harbor adult neural precursors, such as striatal tissue adjacent to the subventricular zone and the cerebral cortex, this gliotic response is accompanied by low-level production of neuronal progeny by cells that closely resemble brain astrocytes (Magavi et al., 2000). These observations have prompted scientists to re-evaluate their perspectives on the roles of brain glia. The classically defined supportive roles of glia were soon amended to include a response during which they re-activate dormant

precursor abilities. When one tried to sort out the meaning of this behavior, a predominant question persistently emerged: is this a *bonafide* ability, including the full potential to repair neural tissue, or is this a remnant progenitor mechanism that will fall short of repair, due to an incomplete regimen of intrinsic progenitor genes and environmental cues? Certainly, the latter seems to be a more pessimistic, yet pragmatic hypothesis, and was the likely answer given the evidence available at the time. Earlier *in vivo* observations revealed very few aspects of RPC function within astroglia, supporting this conclusion. Over the past decade, however, researchers have been able to conclude that adult brain neural precursors do, in fact, exhibit a robust potential for cell replacement.

This same evolution in Müller cell science, and their potential use as a substrate for neural repair, is currently underway. Characterization of their response to injury, exogenous growth factors and changes in intrinsic gene expression have promoted Müller cells up the hierarchy of cytogenic potential. In this thesis, I demonstrate that as seen in adult SVZ NPCs, adult Müller glia also respond to high levels of extracellular EGF. EGF-responsiveness is a keystone feature of adult NPCs, and EGF treatment correlates with the up-regulation of progenitor mechanisms. Although we conclude that EGF is not sufficient to induce the expression of a sufficient panel of progenitor mechanisms required for complete RPC-like function, we do identify EGF as one factor that may be used in conjunction with a regimen of factors for Müller glial re-activation.

Müller Glia are "Restricted Retinal Precursors"

Having established that Müller glia respond to growth factors that are typically used for NPC expansion in brain, up-regulate a subset of phenotypes seen in RPCs, and

re-enter the cell cycle to generate viable progeny, where do these cells reside in our framework of stemness? As was mentioned in the introduction of this thesis, I chose to define true neural stem cells as self-renewing and multipotent, while progenitors and precursors are far more restricted in one or the other of these functional phenotypes. Fred Gage, one of the pioneering scientists in adult neurogenesis, has boldly stated that any cell that divides is a stem cell (personal communication), and essentially, it is our duty to figure out how to customize our approach of manipulation in order to harness that stemness. Our data does not suggest that Müller glia, under the conditions tested here, are multipotent (Figure 4-6). We also did not assay whether or not they retain all of their progenitor features over multiple passages. In fact, we determined that they require multiple passages in order to de-differentate. I also show that our cocktail of growth factors was only able to stimulate a limited regimen of retinal neuronal phenotypes. Given the present status of these data, I would propose that although Müller glia may, someday exhibit robust neurogenic potential, they currently are more appropriately defined as restricted retinal precursor cells.

Therapeutic Cell Restorative Strategy in the Retina: Keeping Simple Perspectives and Goals

Contrasting Responses to Axotomy and Growth Factor Exposure by Müller Glia and Ciliary Body Progenitors: Is the Activation of Multiple Neurogenic Sources Counter-Productive?

We have established that CB progenitors, and not Müller glia, respond to the death of RGCs following ON axotomy (Chapter 2). As discussed above, one would presume that given the aggressive response of Müller glia to damage in lower-vertebrates, and their proximity to RGCs, that they would be the most likely candidates to

respond. As described previously, the relatively mild and protracted nature of damage following ON axotomy in comparison to excitotoxic injury may explain this observation. Of particular interest, however, is the activation of CB progenitors, which reside a relatively long-distance from RGCs when compared to Müller cells. This observation raises the issue of threshold of response by these two candidate precursor populations. When attempting to activate retinal precursors in a therapeutic context, our observation that multiple progenitor pools exhibit variable thresholds of activation raise some important issues. First, how do we manage to manipulate one population without inadvertently activating the other? Second, in the event that we do activate a second population, will the mode of activation of one population elicit an undesired response such as tumorogenicity? I believe the answer to this resides in our capacity to target unique mechanisms in each of these two candidate cell pools. The data presented in this thesis suggests that distinctly different etiologies of progenitor behavior are evident within these two populations of cells. This is a fortunate observation which provides the possibility that unique, and possibly divergent pathways of activation exist in CB progenitors and Müller glia.

Müller Glia May Provide a Substrate for the Replacement of Single Retinal Neuron Subtypes

The restricted nature of Müller cell potential detailed in this thesis leads me to discuss their possible role, if any, in therapeutic cell replacement. To begin, the acute and profound loss in retinal tissue brought upon by severe pathology will not likely be amenable to regeneration via the use of any endogenous NPC. Reasons for this lay in the immense degree of complexity and the fidelity required in every aspect of organogenesis.

A number of retinal diseases, however, are the result of the death of a single retinal cell type. This is an incredibly important fact that provides hope for recovery of function and the therapeutic use of NPCs. This hope is situated in our understanding of the production of single retinal cell types in the retina. As mentioned earlier, it is evident from work in developmental neurobiology that RPCs require a specific "transcriptome" to be intrinsically expressed in order for RPCs to generate the wide array of retinal cell types within the retina. These intrinsic programs exist as a highly dynamic, shifting pattern of expression that is required for the successive production of individual classes of retinal cells. It has been shown that the manipulation of transcription factor expression, either by augmentation of gene expression within RPCs, or their treatment with growth factors, can increase the propensity toward the genesis of individual cell types. In some circumstances, single genes are necessary, although not sufficient, for this switch in retinal subtype specification. As such, it is plausible that in the not-so-distant future, we may be able to stimulate endogenous Müller glia to generate a single cell type within the retina. With respect to my findings, I describe not only a response by Müller glia that resembles de-differentiation, but also a re-differentiation effect brought about specifically in response to the exposure of BDNF and EPO. The production of cells which resemble photoreceptors, both morphologically and phenotypically, suggests that activation of BDNF and EPO pathways may be required for the cell-type-specific production of these cells in the adult. Narrowing the types of cells produced following progenitor reactivation in Müller glia will be imperative, as the surplus production of cells that have not been lost will presumably prove to be redundant. In the event that we are unable to recapitulate all the required RPC mechanisms for Müller cell neurogenic activity, as is the case in this

thesis, forced expression using novel molecular techniques may provide the next step toward filling in gaps in gene expression. Therefore, in the event of selective loss in retinal neurons, it is not unreasonable to speculate that Müller glia may be stimulated to generate a single class of neuron associated with that pathology, for the purposes of therapeutic cell replacement.

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