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Biology of Amacrine Cells and Retinal Ganglion Cells in the Developing Retina

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UNIVERSITY OF MIAMI

BIOLOGY OF AMACRINE CELLS AND RETINAL GANGLION CELLS IN THE DEVELOPING RETINA

By

Noelia J. Kunzevitzky

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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the requirements for the degree of
Doctor of Philosophy

BIOLOGY OF AMACRINE CELLS AND RETINAL GANGLION CELLS IN THE
DEVELOPING RETINA

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How do neighboring neurons differ? How are they similar? How do they relate to each other? Retinal ganglion cells (RGCs) and amacrine cells are born during the same developmental window, from the same population of progenitor cells, and some amacrine cells even migrate to the same retinal layer where RGCs reside, the ganglion cell layer. Amacrine cells are presynaptic to RGCs, whose axons subsequently project through the optic nerve and carry visual information to the brain. Although the cell biology of RGCs has been studied in some detail, relatively little is known about the cell and molecular biology of amacrine cells. What distinguishes these neighboring cell types and in what ways are they similar? Here I present a series of studies examining amacrine cells and RGCs, with a view towards better understanding the development and cell biology of these neurons.

The retina has been long used as a model system to study central nervous system (CNS) development and regeneration: RGCs fail to regrow their axons after injury, and RGC survival is compromised in optic neuropathies such as glaucoma. In contrast, amacrine cells survive even after the loss of their targets, RGCs, in glaucoma and other optic neuropathies.
While the signaling for RGC survival in vitro has been widely studied, little is known about the molecular mechanisms that may underlie amacrine cells' resistance to neurodegeneration.

Taking advantage of our unique method to highly purify amacrine cells away from other retinal neighbors and glial cells, I found that amacrine cells can survive at very low densities in culture and that they do not require addition of exogenous trophic factors, unlike RGCs. Interestingly, blocking of MEK1/2 or PI3K signaling pathways significantly impaired survival, suggesting that these intracellular signaling pathways are necessary for amacrine cell survival. Thus, while amacrine cell and RGC survival seem to be regulated through similar signaling pathways, these two cell types have different requirements for exogenous peptide trophic factors. Because amacrine cells were able to survive in our low density cultures in serum and peptide trophic-free media, it is possible that amacrine cell survival is regulated by autocrine signaling, or by hormones and/or antioxidants. Thus these retinal interneurons may not depend on target RGCs for peptide trophic support.

Amacrine cells are a heterogeneous group of interneurons that modulate retinal signaling of visual information onto RGCs. There are more than 30 subtypes described in the mammalian retina, characterized by myriad morphologies and the secretion of different neurotransmitters.

Despite their apparent inability to differentiate axons and dendrites, purified amacrine cells in vitro extended neurites with varied lengths and
morphologies, raising the hypothesis that the regulation of these processes has an intrinsic component. Specifically, I asked whether purified amacrine cell subpopulations would extend neurites similarly in vivo and in vitro. Surprisingly, three purified amacrine cell subpopulations recapitulated aspects of their in vivo morphology in vitro, consistent with the existence of intrinsic mechanisms of neurite growth and patterning in the developing retina. Thus, I have demonstrated that there is an intrinsic regulatory component that contributes to the varied morphology of amacrine cell neurites found in vivo.

To further characterize differences between amacrine cells and RGCs, I generated a database of amacrine cell gene expression during development and compared it to the transcriptome of RGCs at the same developmental ages. I found ~75% similarity among the genes expressed in RGCs and amacrine cells during development. However, I focused my interest in genes that were differentially regulated because they might underlie amacrine cells’ resistance to neurodegeneration and could help understand the differences in polarity between amacrine cells and RGCs. Comparing the gene expression profiles of these two cell types, I found that RGCs expressed higher levels of the pro-apoptotic molecules Bax and Bad. This raises the interesting hypothesis that amacrine cells may be more resistant to degeneration than RGCs because they do not express as many pro-apoptotic molecules as RGCs do. In addition, I generated a list of polarity-associated candidate genes that are differentially expressed in amacrine cells and RGCs. Together, these data could be combined for
therapeutical purposes. Switching dying RGCs to an amacrine cell-like state may help preserve these cells in neurodegenerative diseases like glaucoma. Conversely, regulating polarity genes in amacrine cells might induce changes in their neurite outgrowth ability that could help understand the mechanisms of cell polarization and axon growth, two critical components to achieve CNS regeneration.

How might these presynaptic amacrine cells influence their neighboring RGCs’ cell biological phenotypes? Previous findings in the laboratory demonstrate that purified RGCs undergo an irreversible loss of their intrinsic axon growth ability during development, and that the process can be signaled by amacrine cells. Thus, amacrine cells are sufficient to signal RGCs to decrease their intrinsic axon growth ability during development. It is not known, however, whether amacrine cells are necessary for this process. I hypothesized that in the absence of amacrine cells, RGCs’ axon growth might be dysregulated in vivo. The creation of the Foxn4\(-/-\) mouse by the Xiang laboratory allowed me to address this question. Foxn4 is a forkhead transcription factor that is required for amacrine cell genesis during retinal development, and as a result the Foxn4 knockout mice have fewer amacrine cells. I found that in the context of a reduced number of amacrine cells, RGCs projected fewer dendrites to the inner plexiform layer in the retina. In addition, RGCs’ axon projection to their target (the superior colliculus) was developmentally delayed, and they failed to penetrate into the retinorecipient layers of the superior colliculus. Finally, Foxn4\(-/-\) mice showed
disrupted optic nerve architecture, albeit the fluorescence intensity of labeled RGC axons in optic nerve cross-sections was similar among animals.

Taken together, these data demonstrate a role for a pre-synaptic partner, amacrine cells, in regulating a neuron's intrinsic axon growth ability and intrinsic patterning.

In conclusion, together these data paint a portrait of how two neighboring retinal cell types differ: amacrine cells are resistant to neurodegeneration whereas RGCs express genes that are associated with apoptosis and glaucoma; amacrine cells do not require the presence of exogenous trophic factors to survive in vitro whereas RGCs do require trophic support. Finally, while amacrine cells and RGCs may differ in their mechanisms for establishing axon and dendrite specification, they both exhibit an intrinsic capacity to grow neurites in vitro that recapitulates their phenotype in vivo. These differences in developmental cell biology may point to new approaches to understanding retinal and retino-collicular patterning, neuronal survival and perhaps even optic nerve regeneration.
To Jeff, my lobster,

and to Mika, our Lechuguita.
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In 2004 I left Argentina and moved to Miami to start graduate school. At that time, my future was a white canvas. Fast forward 5 years, and I am thanking the people that were instrumental in making my life picture perfect. That is the most fascinating aspect about life and science in general: you never know what is going to happen next.

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The lab was a collection of mostly empty benches when I started, but luckily there were two special neighbors at each side of my bench. One of them still honors me with her friendship, after sharing uncountable hours in the cell culture room, reagents, laughs, tears and our love for playing the same CD over and over. Darcie, I think I owe you a copy of “Love Actually” and you are buying the chocolate chip muffins next time. I ended up marrying my other bench neighbor; so needless to say, his strategic location was a great incentive for me to stay in the lab.

I had so much fun in the lab. My experience would not have been the same without you guys. Raul, you lifted my spirit so many times. Thank you for your friendship, for becoming a permanent part of my family, for your enthusiasm
and for teaching me that a café con leche at the right time and in good company is the cure for all evils.

Yoni, I enjoyed getting to know your happy soul. Thank you for opening up to me, and for sharing your dreams and your ghosts. I am so lucky to have you in my life.

Yuanli and Mauris, I miss you!

Kevin, I promise I will never again give you hundreds of amacrine cell images to hand trace on a weekend, and that torturing somebody else with Excel spreadsheets will not feel special at all. I had a blast working with you and I wish you the best of luck in your new endeavors.

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Mika, Mommy will be a doctor soon, but I will always be your mommy first.

Last but not least, Love, I want to thank you for your enormous patience and for always holding my hand. You were right: we now have the sun and the moon.
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Chapter One: This work was submitted for publication on October 2009 to Investigative Ophthalmology and Visual Science (IOVS):


I prepared and wrote this paper based on my work. I am responsible for almost all of the figures in it. M Vanessa Almeida provided technical help for Figure 1.5, and my mentor, Jeffrey Goldberg, contributed to Figure 1.1B, edited the paper and supervised my work.

Chapter Two: This work was submitted for publication on October 2009 to the Journal of Neuroscience:

I prepared and wrote this paper based on my work. I am responsible for all of the figures in it. Kevin Willeford, a Neuroscience undergraduate student working under my supervision, collected data for Figures 2.2, 2.4, 2.5 and 2.6; Bill Feuer performed the statistical analysis in Figures 2.2 and 2.3; M Vanessa Almeida performed the immunostaining in Figure 2.1, and my mentor edited the paper and supervised my work.

Chapter Three: This work will be submitted for publication in 2009 to an appropriate journal:


(2009) Foxn4 is required for normal RGC axon projection to the superior colliculus. (Manuscript in preparation; * denotes first co-authorship.)

I prepared and wrote this paper in collaboration with M Vanessa Almeida based on my research hypothesis. Vanessa collected the data to generate most of the figures. I analyzed all of the data in the paper, except for Fig. 3.1, which was contributed by Yuanli Duan. Yuanli also wrote the corresponding sections in the manuscript (Methods, Results, Discussion). Shengguo Li contributed to Fig. 3.7, and my mentor edited the paper and supervised all the work.
Appendix: This paper was published in 2007 in the Journal of Neuroscience:


* denotes first co-authorship

Jeff Goldberg and the other co-authors collected the samples to perform the microarray hybridization, contributed to the microarray data analysis, wrote most of the manuscript, and generated Figs. 1, 3, 4 and 6. I re-analyzed the microarray data, contributed to Fig. 2, and I am responsible for Fig. 5 and all the Tables including the supplemental material. I also wrote the corresponding sections in the manuscript. Jeff Goldberg finalized the submission of the paper and supervised my work.
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CHAPTER ONE: Amacrine Cell Gene Expression and Survival Signaling: Differences from Neighboring Retinal Ganglion Cells

CHAPTER OVERVIEW

**Purpose.** To describe how developing amacrine cells and retinal ganglion cells (RGCs) differ in survival signaling and global gene expression.

**Methods.** Amacrine cells were immunopurified and processed for GeneChip analysis. For survival studies, purified amacrine cells were cultured at low density in serum-free media with and without peptide trophic factors and survival pathway inhibitors. To analyze the differences in gene expression between amacrine cells and RGCs, we compared the transcriptomes of these two cell types at the same developmental ages.

**Results.** We found that amacrine cells’ transcriptome is very dynamic during development. Amacrine cell gene expression is remarkably similar to that of RGCs, but differs in a number of gene ontologies including polarity- and neurotransmission-associated genes. Unlike RGCs, amacrine cell survival *in vitro* is independent of cell density and the presence of exogenous trophic factors, but nonetheless requires Erk activation via MEK1/2 and AKT signaling. Finally, comparison of the gene expression profile of amacrine cells and RGCs provided a list of polarity-associated candidate genes that may explain the inability of amacrine cells to differentiate axons and dendrites as RGCs do.

**Conclusions.** Comparison of the gene expression profile between amacrine cells and RGCs may improve our understanding of why amacrine cells
fail to differentiate axons and dendrites during retinal development, and of what makes amacrine cells differ in their resistance to neurodegeneration. Switching RGCs to an “amacrine cell-like” state could help preserve their survival in neurodegenerative diseases like glaucoma, and amacrine cells could provide a ready source of replacement RGCs in such optic neuropathies.

CHAPTER INTRODUCTION

Amacrine cells are retinal interneurons essential for visual function, as they modulate retinal signaling onto retinal ganglion cells (RGCs) (Nelson et al., 1981). More than 30 types of amacrine cells in the mammalian retina can be classified by morphology, physiology, stratification patterns or expression of specific markers (Kolb and Nelson, 1981; Kolb et al., 1981; Jeon et al., 1998; Masland, 2001). In the developing retina, amacrine cells are born at the same time as RGCs, and many of them even migrate to the same layer of the retina as RGCs (Lee et al., 1999; Rapaport et al., 2004). Interestingly, amacrine cells appear to resist neurodegeneration after either photoreceptor or RGC death (Kielczewski et al., 2005).

The signaling of RGC survival has been well-studied (Meyer-Franke et al., 1995; Goldberg and Barres, 2000; Goldberg et al., 2002b; Goldberg et al., 2002a); however, little is known about amacrine cell biology. For example, what is the molecular basis for their resistance to degeneration upon loss of their targets (RGCs)? Why don’t they differentiate their neurites into axons and dendrites as RGCs do? Here we characterize amacrine cell biology in vivo and in
vitro, using highly purified cultures of amacrine cells. These data present a comprehensive comparative analysis of two neighboring CNS neurons, and demonstrate fundamental differences from RGCs in amacrine cell gene expression and survival signaling.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were used for these experiments in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in accordance with institutional animal care and use committee review and approval.

Amacrine cell and RGC purification

Amacrine cells and RGCs were purified by immunopanning as previously described (Meyer-Franke et al., 1995; Goldberg et al., 2002a). Briefly, embryonic and postnatal rat retinas were dissociated with papain (Worthington, Lakewood, NJ) and mechanically triturated to obtain a single cell suspension. Enrichment of amacrine cells to 88% purity was achieved after depleting rat macrophages (1:75, #AI A51240, Accurate Chemical, Westbury, NY), T11d7- and Ox7-positive cells (including RGCs), and immunopanning for Vc1.1-positive cells (Fig. 1.1 and Fig. 1.2) (Arimatsu et al., 1987).
RNA preparation, microarray hybridization and data analysis

Amacrine cells from embryonic (E20) and early postnatal (P5, P11) rats were acutely purified (Goldberg et al., 2002a). Total RNA was extracted (RNasey; Qiagen, Valencia, CA) and shipped to the NIH Neuroscience Microarray Consortium (at the University of California in Los Angeles, CA), where it was amplified and processed for hybridization onto rat genome arrays RAE 230 2.0 (Affymetrix, Santa Clara, CA). Three Genechips were used for each postnatal amacrine cell age (P5, P11) and four Genechips for E20 amacrine cells. The starting material for each Genechip was RNA collected from independent samples collected on different days.

Raw data files were analyzed with Microarray Suite 5.0 (Affymetrix, Santa Clara, CA), and subsequent analysis was performed using Excel (Microsoft, Redmond, WA) and NetAffx™ Analysis Center (Affymetrix, Santa Clara) as described in the Results section. Amacrine cell microarray data have been deposited in the NIH Neuroscience Microarray Consortium database, http://np2.ctrl.ucla.edu/np2/home.do.

Comparison of amacrine cell and RGC gene expression profiles

We compared the gene expression profile of amacrine cells to previously published data on RGCs (Wang et al., 2007). Since the microarray platforms used for amacrine cells and RGCs were different, (Affymetrix RAE 230 2.0 and RG34U A-C, respectively), we developed a method for probe set matching. Using the “Array Comparison Spreadsheets” from Affymetrix
(http://www.affymetrix.com/support/technical/manual/comparison_spreadsheets_manual.pdf), we found that of 31099 probe sets in the RAE 230 2.0 array and 26379 probe sets in the RG34U A-C array set, 16749 rat genes were probed by both platforms, allowing us to analyze cross-platform data representing 54% of the amacrine cell probes and 63% of the RGC probes.

One of the caveats of using the Good Match Spreadsheets is that the relation of probe sets between RAE 230 2.0 and RG 34UA-C arrays is not one-to-one, but rather many-to-one (Hwang et al., 2004). We eliminated duplicates in our datasets by removing the probes with the lowest percentage “Present” call (Affymetrix algorithm) across samples and then the lowest expression levels across all ages. Following these criteria, we also removed probes with the same UniGene identification numbers (Kuo et al., 2002), which yielded a final pool of 14457 shared unique probes between the amacrine and RGC datasets. The final probe comparison spreadsheet is available online at the journal's website as Supplemental Table 1.2.

To compare gene expression levels for the full probe set between amacrine cells and RGCs (as in Fig. 1.4), analysis of the gene expression profiles was done independently of expression levels, to avoid the hazards of cross-platform normalization. We generated a ranking system, where the average of 3-4 biological replicates for each probe within one dataset was ranked according to its expression level compared to the rest of the probes within that dataset. The highest expressed probe in amacrine cells received a rank of “1”
and so forth; the same was done for RGCs. Rankings within an ontology were averaged for each cell type at each age studied, creating the index used in Fig. 1.4.

When directly comparing expression levels of a subset of genes (as in Fig. 1.5) we normalized the datasets by comparing the average of 14457 probes for amacrine cells and RGCs at each developmental age. The adjusted RGC absolute expression levels were calculated by multiplying the RGC expression levels by a ratio factor generated when dividing the normalized, average expression level of all amacrine cell probes by the normalized, average expression level of all RGC probes.

**Immunofluorescence**

For quantification of the purity of amacrine cell cultures, amacrine cells were acutely purified, plated on PDL-coated glass coverslips and immunostained at 1 DIV. Briefly, cells were fixed with 4% PFA for 5 minutes, rinsed three times in PBS, and permeabilized for 5 minutes with 1% Triton X-100. After another round of rinses in PBS, cells were blocked and permeabilized with 20% donkey serum and 0.1% Triton X-100 in antibody buffer (150mM NaCl, 50mM Tris base, 1% BSA, 100mM L-Lysine, 0.04% Na azide, pH 7.4). Sheep anti-Chx10 (1:200, #AB9016, Millipore, Billerica, MA) and mouse anti-Syntaxin (1:200, #ab3265, Abcam, Cambridge, MA) antibodies were incubated overnight at 4°C. Secondary antibodies (Donkey anti-mouse Alexa 488 and donkey anti-sheep Alexa 594; Invitrogen, Carlsbad, CA) were used at a dilution 1:500 and incubated for 4 hours
in the dark at room temperature. Coverslips were later mounted on glass slides using Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and examined with a Zeiss inverted fluorescence microscope.

For immunostaining of polarity genes, RGCs and amacrine cells acutely purified from the same animals were cultured on PDL-coated glass coverslips with or without laminin (Trevigen, Gaithersburg, MD) respectively, in serum-free media. At 3DIV, cells were incubated at 37°C with PFA (2% final concentration) for 30 minutes, post-fixed with 4% PFA for 10 minutes at room temperature and the coverslips were rinsed three times with PBS and blocked for 30 minutes with 20% normal goat serum and 0.2% Triton X-100 in antibody buffer. Primary antibodies were incubated overnight at 4°C as follows: rabbit anti-Par6 (1:100, #sc-25525, Santa Cruz Biotechnology, Temecula, CA), rabbit anti-atypical PKC (1:100, #sc-216, Santa Cruz Biotechnology, Temecula, CA), mouse anti-Limk1 (1:500, #L13020, BD Biosciences, Mississauga, ON Canada) and mouse anti-stat3 (1:100, #9139, Cell Signaling Technology, Danvers, MA). Secondary detection was performed using fluorescent antibodies at a 1:500 (Alexa-488; Invitrogen, Carlsbad, CA). TRITC-conjugated phalloidin (3.3ug/ml; #P1951 Sigma, St. Louis, MO) was added together with the secondary antibodies and incubated overnight at 4°C. The coverslips were mounted on glass slides and examined with a Zeiss inverted fluorescence microscope. Cells immunostained with the same antibody were exposed to the same fluorescence intensity.
Exposure was carefully controlled to maximize comparison between amacrine and RGC immunofluorescence and to better capture the expression of polarity genes in the neurites.

**Amacrine cell culture and survival assays**

Acutely purified amacrine cells were cultured at different densities (8, 47 or 156 cells/mm²) in tissue-culture wells coated with PDL (70 kDa, 10 µg/ml; Sigma) in serum-free Neurobasal media (Gibco, Carlsbad, CA) as described (Meyer-Franke et al., 1995; Goldberg et al., 2002a) containing insulin (50 ng/ml), forskolin (5 mM; Sigma), CNTF (10 ng/ml), BDNF (50 ng/ml; Peprotech, Rocky Hill, NJ) and a modified version of B27,(Chen et al., 2008) with antibiotics (1X Pen-Strep; Gibco, Carlsbad, CA). Cultures were maintained at 37°C in a humidified incubator with 10% CO₂. To evaluate cell survival as a function of the cell density (Fig. 1.6A), we used calcein-AM (1 uM; Invitrogen, Carlsbad, CA) and the nuclear dye DAPI. Initially, we intended to use DAPI as a nuclear maker for all cells and calcein as a marker of living cells. This would allow unequivocal counting of cell viability. To our surprise, when imaging the cells under a fluorescence microscope, we saw exclusion of DAPI by calcein-positive cells. We then added a third dye (propidium iodide) and confirmed this result. Invitrogen states that DAPI is cell impermeant when added at low doses. We used DAPI at a final concentration of 30 ng/ml. Cells that were calcein⁺/DAPI⁻ were considered to be alive; dead cells were DAPI⁺. For the rest of the survival experiments (Fig. 1.6B, C), cells were plated at the lowest density (8 cells/mm²). We used calcein-
AM (1 µM) and the nuclear dyes Hoechst (1:5000; Invitrogen, Carlsbad, CA) and propidium iodide (PI; 1:5000; Sigma, St. Louis, MO) were added to the media for 30-45 minutes. Live cells were defined as calcein+/PI–; all PI+ cells were counted as dead. Cells were manually counted, and at least 3 wells per condition were averaged within each experiment. Experiments were repeated at least 5 times to confirm results.

**Immunoblotting and densitometric analysis**

Acutely purified amacrine cells were resuspended in serum-free media with or without various peptide trophic factors and pharmacologic inhibitors, and rotated for 2 hours at 37 °C, after which cells were processed for protein extraction following standard protocols. The pharmacologic inhibitors used were UO126 (10 µM), K252a (400 nM), AG490 (100 µM) and PD98059 (20-30 µM; Calbiochem, San Diego, CA) and LY294002 (50 µM; Cell Signaling Technology, Danvers, MA). Antibodies used for Western blot were: rabbit anti-phospho-Mapk antiserum (1:1000; #E7028, Sigma, St. Louis, MO), goat anti-Erk1 antiserum (1:1000; #sc-93, Santa Cruz Biotechnologies, Santa Cruz, CA), rabbit anti-phospho-Akt antiserum (1:1500; #9271), and rabbit anti-Akt antiserum (1:1000; #9272 Cell Signaling Technology, Danvers, MA). Secondary antibodies conjugated to horseradish peroxidase (1:2000; Santa Cruz Biotechnologies, Santa Cruz, CA) were incubated for 2 hrs at room temperature and revealed with ECL (Pierce Biotechnology, Rockford, IL). Densitometric analysis of the western
RESULTS

Amacrine cell purification

Amacrine cells from E20 and P8 rats were acutely purified by immunopanning using the Vc1.1 antibody (Barnstable et al., 1985) and cultured in serum-free media (Fig. 1.1A). Since amacrine cells only make up to 9-12% of the cells in the retina (Goldberg et al., 2002a) and ~40% of the cells in the inner nuclear layer of the mouse retina (Young, 1985; Jeon et al., 1998), we performed sequential immunopanning to obtain a high amacrine cell yield (Fig. 1.1A) (Goldberg et al., 2002a). To confirm the purity of the cultured cells, we performed double immunostaining using both the monoclonal antibody HPC-1 (Fig. 1.1B), which recognizes syntaxin, an amacrine cell marker (Bennett et al., 1992; Tagaya et al., 1995) and an antibody against the pan-bipolar cell marker Chx10 (Liu et al., 1994). We found that 88% of the purified cells were immunopositive for HPC-1 and 7% were immunopositive for Chx10, while no cells were immunoreactive for both antibodies (Fig 1.2). Thus, the immunopanning technique yielded a culture highly enriched in amacrine cells, although data from postnatal amacrine cell cultures does reflect a small contamination from bipolar cells.
Amacrine cell gene expression profile

What genes do amacrine cells express during development? Recent analyses of amacrine cell gene expression at the single cell level have yielded beautiful pictures of the molecular diversity of these cells (Trimarchi et al., 2007; Cherry et al., 2009), but we had undertaken an overview of amacrine cell gene profiling at a population level through perinatal development. mRNAs isolated from acutely purified amacrine cells from E20, P5 and P11 rats were hybridized to Affymetrix Rat Genome 230 2.0 expression arrays containing 31,099 probes representing more than 28,000 rat genes. 3-4 biological replicates were collected for each age, and we found little intersample variability within the same age group (Pearson r^2>= 0.90 for all pairwise comparisons) (Fig. 1.3A).

We found that amacrine cells express 21899 (70%) of the 31099 probes in at least 2 or more of the samples at one or more ages (Fig. 1.3B), and 16247 probes (52%) are expressed at all ages, using Affymetrix’s “Present” call algorithm. Of those 21899 probes present in at least 2 or more of the samples at one or more ages, 2468 probes (11%) changed at least 3-fold during development, 344 (2%) changed at least 10-fold and 120 (0.5%) changed at least 20-fold (Fig. 1.3C and Table 1.1). Using ANOVA to calculate probes that changed more than 3-fold with a p-value less than 0.05, 2231 probes (~10%) changed between E20 and P11 (without correction); 374 probes (1.7%) changed if a Bonferroni correction is used (Fig. 1.3B). These represent two extremes of statistical validation; a “true” number of changing probes likely lies between these two numbers.
To further classify the gene expression profile of amacrine cells, we distributed the 31099 probes of the RAE 230 2.0 arrays into overlapping gene ontology categories using data available from Affymetrix’s Netaffx Analysis Center (http://www.affymetrix.com/analysis/index.affx) and the Gene Ontology Consortium database (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) (Ashburner et al., 2000). We fit 16753 probes (54%) into 27 partially overlapping categories, and we found that amacrine cells express 52-89% of the probes in each category. Categories such as “mitochondria,” “cell cycle,” “chromosome,” and “ubiquitin” were over-represented and the category “G-protein” was under-represented (Chi-Square; Fig. 1.3D). We next analyzed the fraction of probes within each category that changed at least 3-fold during development. Interestingly, we found that 11 out of the 27 categories were significantly regulated during development (Chi-Square; Fig. 1.3E), including “neurotransmission/tter,” “plasma membrane”, and “migration.” These results may reflect the vast differentiation that amacrine cells undergo between E20 and P11 (Casini and Brecha, 1992; Redburn and Rowe-Rendleman, 1996; Johnson et al., 2003; Kay et al., 2004; Emanuela et al., 2009), when they start secreting neurotransmitters and there are changes in the levels of their receptors on the plasma membrane. At this same developmental stage, amacrine cells migrate to populate the INL and GCL (Prada et al., 1987). Conversely, probes in categories such as “cell cycle,” “polarity” and “apoptosis” were less likely to change during development, as the peak of amacrine cell generation occurs embryonically (Young, 1985; Reese and Colello, 1992; Cepko et al., 1996; Rapaport et al.,
2004; Emanuela et al., 2009). It is possible that some of the probes in these categories are also changing at the protein level during development by post-translational regulation. Compartmentalization, binding with inactivating partners or changes in the phosphorylation levels are not reflected in these microarray analyses (Fig. 1.3E and Table 1.2). These data suggest that the amacrine cell transcriptome is very dynamic during development, similar to our prior findings with purified RGCs (Wang et al., 2007).

Changes in amacrine cell gene expression could be due in part to a change in the subpopulations of amacrine cells purified by immunopanning at embryonic and postnatal ages, although we have yields of well over 50%, suggesting that we are purifying a broad swath of amacrine cell subtypes at all of these ages. To further validate our dataset, we compared our E20 amacrine cell data against recently published P0 amacrine cell and RGC single-cell expression profiling (Trimarchi et al., 2007; Cherry et al., 2009). Consistent with their results, we found that amacrine cells (and not RGCs) express high levels of the transcription factor AP-2 beta (TCFAP-2β) and that all the samples of amacrine cells expressed high levels of neuronal leucine-rich repeat protein-3 (Lrrn3), two recently validated amacrine cell markers (Trimarchi et al., 2007). We also observed amacrine cell expression of probe sets for gamma synuclein (Sncg), early B-cell factor 3 (Ebf3) and neurofilament 68 (Nefl), genes previously described to be highly enriched in RGCs (Mu et al., 2004; Mu et al., 2005; Surguacheva et al., 2008). These discrepancies may be explained by a number of experimental differences, including post-transcriptional control of expression (not
explored here), neuronal age and species (E20 rat versus P0 mouse), starting RNA material (millions of purified amacrine cells including different subtypes pooled together versus single cells analyzed separately), potential amplification artifacts (less amplification is required for pooled cells), and the potential for contamination or cellular mis-identification in either dataset.

Comparison of amacrine cell and RGC gene expression through development

We next compared our gene expression datasets of amacrine cells and RGCs (Wang et al., 2007). We identified 14457 probes shared between the different GeneChip platforms of the two datasets, over half of the total data. The manipulation necessary for this comparison across different platforms (see Methods) may have decreased the robustness of these analyses, but the large number of shared, cross-platform probes would be expected to compensate for this limitation. Of the 14457 probes shared between the two array types (see Methods; Fig. 1.4A), 8575 (59%) were “present” by Affymetrix algorithm in at least 2 samples of one age in both genechip datasets; 2640 (18%) were present only in amacrine cells, and 432 (3%) were expressed only in RGCs; the remaining 2910 (20%) were absent from both cell types. A breakdown of these numbers at E20, P5 and P11 is shown in Fig. 1.4A. Of the 3072 probes expressed at any age either by amacrine cells or by RGCs but not by both cell types, amacrine cells expressed more “unique” genes than RGCs at all ages (Fig. 1.3A) and across all 35 gene ontologies analyzed. Interestingly, we found 25 neurotransmitter-associated genes that were exclusively expressed by
amacrine cells at all ages but not by RGCs (Table 1.2) and only 2 specific to RGCs. Occasionally probes had higher absolute expression levels in RGCs than in amacrine cells, but the Affymetrix algorithm called them “absent,” as commonly occurs when analyzing probes with low absolute levels of expression. The higher number of neurotransmitter-associated probes expressed exclusively by amacrine cells and not RGCs is consistent with the transmitter variety described in amacrine cells as a whole (Massey and Redburn, 1987; Kolb, 1997; Masland, 2001; Wassle, 2004).

We next compared the gene expression levels for the probes expressed by both RGCs and amacrine cells at different ages (Fig. 1.4A). Since the absolute gene expression levels for amacrine cells and for RGCs are on different, arbitrary scales, we converted absolute gene expression levels to ranked gene expression levels for each cell type, assigning the gene with the highest amacrine cell expression at a given age a rank of 1, and continuing down to a rank of 10525 for E20, 10574 for P5 and 10502 for P11 (the numbers of genes present at each of those ages), and repeating this process for RGC genes. Since RGCs expressed fewer genes at every age than amacrine cells, the RGC rank list was scaled to 10525 for E20, etc, to match the length of the amacrine cell gene list. The genes “Present” only in amacrine cells were given terminal rankings (e.g. 10526) in RGCs, and vice versa, at each age. We then compared the ranked expression for each gene between amacrine cells and RGCs, to create a simple index. The average of the differences between rankings of amacrine cells and RGCs compared at E20, P5, and P11 of all the
probes within a given gene ontology is shown in Fig. 1.4B, where the scale shows genes ontologies with genes that are more highly expressed in RGCs at one end, and more highly expressed in amacrine cells at the other end. For example, RGCs at all ages expressed higher levels of genes within the “neurotransmi-” and “immune” ontologies (top of Fig. 1.4B and Table 1.2). This difference could be due to the fact that amacrine cells secrete a variety of neurotransmitters and RGCs only a few but at higher levels, thus demonstrating greater differences in gene expression rankings (Massey and Redburn, 1987; Kolb, 1997; Masland, 2001; Wassle, 2004). On the bottom of Fig. 1.4B, amacrine cells expressed higher levels of genes in the categories “chromatin,” “DNA” and “transcription.” The index identified differences between amacrine cells and RGCs that were largely consistent across E20, P5 and P11 ages in most ontologies, except for a few ontologies such as “polarity,” “neurit-,” “chromosome,” and “neurotransmi-” which demonstrated greater developmental variability across this age range between these two neuron types.

Comparing the expression profile of more than 50 polarity-associated genes, we found that a number are differentially regulated during amacrine cell development, or differ between amacrine cells and RGCs (Table 1.3 and Fig. 1.5B, D, F, H). For example, the microtubule affinity-regulating kinase 2 (Mark2), which negatively regulates dendrite development in cultured hippocampal neurons (Terabayashi et al., 2007), was expressed at moderate levels by amacrine cells but not detected in RGCs. Conversely, signal transducer and activator of transcription 3 (Stat3), which when activated can promote neurite
outgrowth in primary sensory neurons (Miao et al., 2006), was expressed 5-fold more in RGCs than amacrine cells (Fig. 1.5B). Consistent with previous findings (Muller et al., 2007), RGCs were immunopositive for Stat3, but we found that amacrine cell immunostaining for Stat3 was barely detectable above background (Fig. 1.5A), consistent with the microarray data (Fig. 1.5B).

We next investigated the expression and localization of a few other “polarity” proteins between the two cell types during neurite growth in vitro. All immunostaining performed trended in the same direction as the microarray data, adding confidence to that data’s reliability. We did not detect any differences in the expression or localization of some proteins including Par6 (Fig 1.5C). Others, however, were differentially expressed or localized. For example, atypical protein kinase C (aPKC), which is a part of the Par3/Par6/aPKC complex required to establish cell polarity in mammalian epithelial cells (Nishimura et al., 2004), and whose activity is necessary for neurite polarization and axon formation in hippocampal neurons (Shi et al., 2003), was localized in the cytoplasm and along initial neurite segments in RGCs, but it was only present in the cytoplasm of amacrine cells (Fig 1.5E). Limk1, a kinase that regulates actin cytoskeleton dynamics (Matsui et al., 2002; Rosso et al., 2004) and controls growth cone motility (Endo et al., 2003), was highly localized to the growth cones in RGCs and also present in the lamellipodia of amacrine cells (Fig. 1.5G). Interestingly, while in RGCs Limk1 has a ubiquitous distribution in the cell body; it is clearly excluded from the nucleus in amacrine cells, which may suggest a difference in its biological functioning between these two cell types (Yang and Mizuno, 1999).
While the functional role of these expressed polarity genes in amacrine cells remains to be tested, taken together, these data suggest that amacrine cells express many of the genes important in neurite differentiation (that is, axon versus dendrite differentiation), and that differential protein localization may explain the differences in axon/dendrite polarization in these cell types.

**Amacrine cell survival signaling pathways**

It is a general tenet in neuroscience that neurons are dependent on their targets for survival, both during development and in the adult (Goldberg and Barres, 2000). In vivo in optic neuropathies, however, amacrine cells appear to resist degeneration after RGC death (Kielczewski et al., 2005). While the molecular signals sufficient to promote RGCs’ survival have been extensively studied and characterized (Mey and Thanos, 1993; Mansour-Robaey et al., 1994; Meyer-Franke et al., 1995; Peinado-Ramon et al., 1996; Klocker et al., 1997; Cui et al., 1999; Goldberg et al., 2002a; Schmeer et al., 2002), little is known about the cues that regulate amacrine cell survival. We analyzed survival-related genes with gene ontologies containing “apoptosis,” “MAPK,” “PI3K,” and a few hand-picked additional genes. We identified the subset of these genes that differed between amacrine cells and RGCs. Of 112 probes found in both datasets, 84 probes (75%) were “present” both in amacrine cells and RGCs, 19 probes (17%) were “present” only in amacrine cells and 7 probes (7%) were unique to RGCs. A number of interesting candidate genes differed, including ciliary neurotrophic factor receptor (Cntfr) and huntingtin (Htt), which were
detected in amacrine cells but not in RGCs. Htt may play a role in survival signaling by enhancing BDNF axonal transport (Gauthier et al., 2004), and is one of the binding partners of optineurin (Faber et al., 1998), a protein in which mutations have been associated with primary open angle glaucoma (Rezaie et al., 2002). While optineurin expression in RGCs increases during development (Rezaie and Sarfarazi, 2005; De Marco et al., 2006; Wang et al., 2007), it would be interesting to explore whether deficiency of its binding partner Htt may underlie RGCs’ susceptibility to neurodegeneration. Among the many genes expressed by both cell types were most of the bcl-2 family. However, RGCs expressed higher levels of the pro-apoptotic molecules Bax and Bad, raising the hypothesis that RGCs are more susceptible to death than amacrine cells because they express less bcl-2 family members, which may make RGCs more prone to apoptosis (Wang et al., 2007).

Do the same signaling pathways mediate survival of RGCs and amacrine cells? To address this question, we purified primary amacrine cells from P8-P9 rats, cultured them in a variety of defined growth media without serum for up to 3 days, and quantified their survival using a live/dead assay (see Methods). First, we found that amacrine cell survival was not enhanced by increasing cell density (Fig. 1.6A), in contrast to RGC survival (Meyer-Franke et al., 1995), suggesting that amacrine cells may not secrete factors into the media that enhance their own survival in a paracrine fashion. Exogenous addition of brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF), two of the trophic peptides that most strongly support RGC survival (Meyer-Franke et al., 1995; Jo et al.,
1999), had no effect on amacrine cell survival (Fig. 1.6B). Indeed, at cell culture densities that are likely too low for amacrine cells to condition the media with paracrine signaling molecules, amacrine cell survival was remarkably high even in the absence of peptide trophic factors. There was a modest effect of adding a modified B27 supplement containing hormones and antioxidants (Chen et al., 2008), particularly in the absence of other peptide trophic factors (Fig. 1.6B). This high, constitutive survival was observed solely from the constituents of the Sato supplement (Bottenstein and Sato, 1979), which includes putrescine, transferrin, progesterone, selenite, and albumin, plus pyruvate, glutamine, thyroid hormone, and n-acetyl cysteine (Fig. 1.6B). These data suggest that amacrine cells may depend more on hormone and antioxidant signals than on peptide trophic factors for their survival.

To explore what intracellular signaling pathways could be mediating the observed survival in vitro, we cultured amacrine cells in “full Sato” (FS) medium containing pharmacological inhibitors for a subset of known survival signaling pathways. When amacrine cells were treated with the MEK1/2 specific inhibitor UO126, there was a 3-fold decrease in survival (Fig. 1.6C). PI3K and JAK/STAT inhibitors (LY294002 and AG490, respectively) also decreased amacrine cell survival in vitro (Fig. 1.6C). Interestingly, although amacrine cells expressed TrkA, -B, and -C by microarray analysis, addition of the Trk inhibitor K252a did not affect survival, consistent with the modest effect of exogenous BDNF on survival in these experiments (Fig. 1.6B, C), suggesting that autocrine Trk signaling is not responsible for amacrine cell survival. We confirmed the
specificity of the UO126, K252a and LY294002 inhibitors by Western blot using amacrine cells acutely purified and allowed to recover for 2hrs at 37°C without peptide trophic factors in the presence of the inhibitors (Fig 1.6D, E). Again, addition of K252a did not affect ERK1/2 signaling compared to control, consistent with its minimal effect on survival. The addition of either UO126 or LY294002 inhibited the activation of ERK1/2 and AKT respectively and impaired amacrine cell survival (Fig 1.6D, E). Taken together, these results show that amacrine cells require MAPK, PI3K and JAK/STAT activation for survival, but we do not know what upstream signals are responsible for activating these pathways.

DISCUSSION

Amacrine cells' transcriptome changes during early retinal development

We and others have previously reported on the transcriptomes of identified cell populations in the CNS, including RGCs, striatal and corticospinal motor neurons (Arlotta et al., 2005; Lobo et al., 2006), and genetic labeling techniques have considerably expanded the scope of CNS neurons amenable to such isolation and analysis, either by cell-sorting fluorescent cells (Sugino et al., 2006) or by directly purifying translated RNAs from neuronal subpopulations that express RNA binding proteins (Heiman et al., 2008). Here we highly purify amacrine cells by immunopanning and provide a database for the transcriptome of amacrine cells at three developmental ages (E20, P5 and P11). Previous datasets have been generated looking at the developmental changes in neighboring neurons' gene expression (Arlotta et al., 2005; Lobo et al., 2006),
and this database, together with the RGC transcriptome (Wang et al., 2007), yields a comprehensive description at a molecular level of two synaptically, closely related CNS neurons. In addition, these data add significantly to the snapshot of single cell gene expression profiling performed on 32 amacrine cells mostly from P0 and P5 (Cherry et al., 2009). With at least 25-30 different morphologic subtypes of amacrine cells previously described (MacNeil and Masland, 1998; MacNeil et al., 1999), a population-level profile of amacrine cell gene expression certainly adds value to the single cell data currently available. These data may provide opportunities in the future for better understanding retinal development, wiring, and degeneration, and ultimately CNS neurological diseases.

Amacrine cells and RGCs demonstrate unique patterns of gene expression

Two cells that come from a common progenitor, migrate (in part) to the same retinal layer, and synaptically integrate in the same retinal layer, may be expected to share common gene expression. Indeed, we found that most genes (>74%) were expressed at similar levels in RGCs and amacrine cells at embryonic and postnatal ages. With an interest in pursuing the observed differences in the cell biology of amacrine cells and RGCs, however, here we focused mainly on their differences in gene expression.

We found that RGCs more highly expressed genes associated with the terms “immune,” “neurotransmitter/mission” and metabolic pathways, such as “Nadh/Nadph.” The role of molecules of the immune system in the CNS was first
thought to be limited to inflammation, injury and autoimmune disorders. Recent findings, in particular in the visual system, have shown that major histocompatibility complex (MHC) class 1 molecules and proteins of the complement cascade like C1q and C3 have an unexpected role in CNS development and plasticity (Huh et al., 2000; Goddard et al., 2007; Stevens et al., 2007). Our data provide a significant list of “immune system genes” expressed by both amacrine cells and RGCs to help build hypotheses for the molecular pathway of synapse formation and elimination during development and after injury.

Similarly, the high expression levels of genes associated with ontologies such as DNA, chromatin and transcription by amacrine cells compared to RGCs could underlie amacrine cell heterogeneity or plasticity during retinal development. With a more dynamic chromatin remodeling capacity, amacrine cells may express more varied sets of transcription factors or other downstream targets at different times, which could contribute to their cellular diversity.

**Amacrine cells’ resistance to neurodegeneration**

Both CNS and PNS neurons are strongly dependent on target-derived peptide trophic signals for their survival, and competition for target-derived signals is intimately related to neuronal survival and synapse formation during development (Purves, 1988; Oppenheim, 1991). Most of the data for these observations has been derived from studies of long projection neurons; it is not known whether the same principles apply to locally integrated interneurons. In
optic neuropathies like glaucoma, when most of the RGCs die, amacrine cells are largely not affected, despite the loss of these synaptic targets (Jakobs et al., 2005; Kielczewski et al., 2005), although most of amacrine cell targets are other amacrine cells (Dowling and Boycott, 1965; Dubin, 1970; Marc and Liu, 2000). Furthermore, in rat optic nerve transection experiments in which over 90% of the RGCs die within 2-4 weeks, amacrine cells continue to survive and express calbindin, calretinin, and GABAergic cell markers after 3 months (Kielczewski et al., 2005). Similarly, GABAergic amacrine cell subpopulations are resistant to retinal ischemia in a rat ischemia/reperfusion model (Dijk et al., 2004).

Previous studies suggested that amacrine cells require high concentrations of insulin for survival (Politi et al., 2001), or that antagonizing TrkB receptor signaling in retinal explants decreases the number of nuclei in the inner nuclear layer (Cusato et al., 2002) and, in vivo, reduces the number of parvalbumin-positive amacrine cells in the retina (Rickman and Bowes Rickman, 1996). Interpretation of such experiments may be confounded by a decrease in the expression of phenotypic cell markers without cell death (Rickman and Bowes Rickman, 1996; Cellerino et al., 1999). Using our ability to highly purify and culture amacrine cells in defined, serum-free media we asked whether the same trophic factors that strongly promote RGC survival (Meyer-Franke et al., 1995) similarly promote amacrine cell survival in vitro. We found that peptide trophic factors did not significantly contribute to amacrine cell survival in vitro even when amacrine cells were cultured at clonal density, despite expression of trkB, CNTF-Ra, LIF-R, and IGF-R1 detected on the microarrays. Interestingly,
blocking of MEK1/2 or PI3K signaling pathways significantly impaired survival, suggesting that these intracellular signaling pathways are necessary for amacrine cell survival, and raising the question of how these pathways are being activated. The Western blot experiments in Figure 1.6 suggest that without the addition of exogenous peptide trophic factors, there is a basal activity of MEK1/2 and PI3K that is further increased when cells are grown in Sato-supplement containing medium. In RGCs, MAPK, PI3K and JAK/STAT signaling pathways are important for survival and axon regeneration (Goldberg and Barres, 2000; Nakazawa et al., 2002; Lingor et al., 2008).

Taken together, these data demonstrate that while RGC and amacrine cell survival are mediated through the same intracellular signaling pathways (Goldberg et al., 2002b), their requirement for exogenous peptide trophic factors differs. Survival of amacrine cells in our low density cultures in the absence of exogenous peptide trophic factors suggests potential regulation either by autocrine signaling (Acheson et al., 1995), or by hormone or antioxidant support alone. Thus these retinal interneurons may not depend on target RGCs for peptide trophic support.

**Can amacrine cell properties be used in the CNS for repair and regeneration?**

To overcome the failure of adult mammalian CNS regeneration, neurons need to survive and reextend their axons to their targets. It is intriguing to consider whether amacrine cells, which exhibit some survival independence from peptide trophic factors and which survive degenerative diseases affecting RGCs,
may represent a residual retinal population of neurons that could be induced to replace the visual functions of lost RGCs. Understanding the molecular components that could allow amacrine cells to extend and differentiate axons may allow these cells to serve as a local source of RGC replacement in designing therapies targeted to cure blindness.
Figure 1.1 Purification of amacrine cells by immunopanning. (A) Acutely dissected retinas from embryonic and early postnatal rats were dissociated in papain and triturated to obtain a single-cell suspension. After depleting the suspension of macrophages and RGCs, amacrine cells were selected using Vc1.1 monoclonal antibody. Subsequent trypsinization yielded amacrine cells at least ~88% pure, which were subsequently cultured in serum-free media. (B) Immunostaining of purified amacrine cells after 2 days in vitro using anti-syntaxin antibody reveals a typical pattern of neurite outgrowth.
Figure 1.2 Immunostaining and quantification of amacrine cell culture purity. P7 amacrine cells were acutely purified and plated on glass coverslips in serum-free media. At 1DIV cells were immunostained for the amacrine cell marker syntaxin (HPC-1) and the bipolar cell marker Chx10. The table shows the percentage of cells that were immunopositive for each marker and represents the average of 3 coverslips (n>90 cells per coverslip).

<table>
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<th>Syntaxin(+) cells</th>
<th>Chx10 (+) cells</th>
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**Figure 1.3** Amacrine cells' gene expression profile. Amacrine cells from E20, P5 and P11 rats were purified by immunopanning. Three to four biological replicates were independently processed for Genechip analysis using Affymetrix RAE 230 2.0 arrays. Data were analyzed using Microarray Suite 5.0 (Affymetrix), National Center for Biotechnology and Information (NCBI), Gene Ontology databases and Excel (Microsoft). (A) Shown is an example scatter plot of gene expression data from two biological replicates of E20 amacrines, demonstrating high replicability. (B) Amacrine cells express ~70% of the 31099 probes in the GeneChips. Of those, 2231 probes change at least 3-fold during development. The number of probes that change significantly decreases after Bonferroni correction ($\Delta =$“change”). (C) Histogram representing frequency of fold-changes in the dataset. Of the 21899 present probes in at least one age, ~10% are developmentally regulated and ~2% change at least 10-fold. (D-E) We classified 31099 probe sets by Gene Ontology and fit 16753 probes (54%) into 27 partially overlapping categories. Amacrine cells expressed 52-89% of the probes in each category (D); the fraction of probes in these gene ontologies that changed >= 3-fold during amacrine cell development is shown in (E) (* significantly higher or lower expressed (in D) or developmentally changing (in E) ontology, by pairwise Chi-Square test at p<0.05 level after Bonferroni correction).
Figure 1.4 Differentially expressed gene ontologies between RGCs and amacrine cells. (A) Venn diagrams describing gene representation on the two different platforms (top) and gene expression at E20, P5 and P11 between amacrine cells and RGCs. After eliminating duplicate probes for the same gene (see Methods), 14457 probes were represented in both datasets. Of these, most were “Present” in both RGCs and amacrine cells at all 3 ages, but over 2600 were expressed uniquely by amacrine cells, and over 300 were expressed uniquely by RGCs, comparing at each age. The area of the circles is not exactly proportional to the number of probes they contain. (B) Average difference in ranked expression between amacrine cells and RGCs in 35 overlapping gene ontologies. Only probes that were “Present” in both datasets were included in the analysis. (See Methods and Results for average ranking details; the datasets and the ranking for each probe can be found online at the journal's website as Supplemental Table 2.)
Figure 1.5 Immunostaining of “polarity”-associated genes and corresponding microarray expression levels. (A,C,E,G) Acutely purified amacrine cells and RGCs were cultured on glass coverslips and immunostained at 3DIV using antibodies as labeled, and rhodamine-conjugated phalloidin to counterstain actin filaments and highlight growth cones. Scale bar, 20 µm. (B,D,F,H) Levels of expression of “polarity”-associated probes from the microarray data for amacrine cells and RGCs during development. Expression levels were adjusted between the two datasets by multiplying the RGC probes by the ratio of the means for amacrine cells and RGCs at each developmental age (see Methods).
Figure 1.6 Amacrine cell survival in vitro. (A) Amacrine cells were purified and plated at different densities in serum-free media. Cells were counted at 1, 2 or 3 DIV after adding calcein-AM and the nuclear dye DAPI. Survival was calculated as the percentage of cells that were calcein (+) of the total number of calcein (+) and DAPI (+) cells. (N=2; n=150 for each condition. Error bars: SEM).

(B-C) Acutely purified P8 amacrine cells were plated at low density in growth media containing peptide trophic factors and forskolin, with and without pharmacological inhibitors (see below). Survival was quantified at 3 DIV using calcein/PI (see Methods) (N>3; n>150 for each condition; ***p<0.001, one-way ANOVA with Tukey post-hoc; **p<0.01, *p<0.05, paired t-test. Error bars: SEM).

(D-E) Amacrine cells were purified and incubated for 2 hrs in growth media with or without inhibitors as labeled, after which they were centrifuged and processed for protein extraction. Example westerns are shown; graphs are the average of at least 2 experiments, normalized to NB Sato (*p<0.05, **p<0.005; unpaired t-test. Error bars: SEM). Abbreviations: B= BDNF; C= CNTF; F= Forskolin; I= Insulin; NB= Neurobasal + penicillin/streptomycin; NB Sato= Neurobasal + Sato stock + pyruvate + penicillin/streptomycin; GM= Growth Media; UO= UO126; K= K252a; LY= LY294002; A= AG490; D= DMSO; FS= Full Sato.
### Table 1.1 Largest gene changes during amacrine cell development.

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*Max*= maximum expression level at all ages; *Min*= lowest expression level at all ages
Table 1.2 "Neurotransmiter" genes in amacrine cells and RGCs.
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CHAPTER TWO: Amacrine Cell Subtypes Differ In Their Intrinsic Neurite Growth Capacity

CHAPTER OVERVIEW

Amacrine cell neurite patterning has been extensively studied in vivo, and more than 30 subpopulations with varied morphologies have been identified in the mammalian retina. It is not known, however, whether the complex amacrine cell morphology is determined intrinsically, or is signaled by extrinsic cues, or both. Here we purified amacrine cells away from their retinal neighbors and glial-derived factors to ask questions about their intrinsic neurite growth ability. Surprisingly, the three subpopulations of amacrine cells studied in vitro recapitulated quantitatively and qualitatively the varied morphologies they have in vivo, suggesting that intrinsic factors contribute to the regulation of neurite patterning.

CHAPTER INTRODUCTION

Amacrine cells are a heterogeneous group of retinal interneurons with a widely varied and complex neurite patterning. There are more than 30 morphologic subtypes described in the mammalian retina (Kolb et al., 1981; MacNeil et al., 1999; Masland, 2001), which range from having few to multiple neurites that may include short or long, axon-like processes, or thin or bushy dendritic-like trees. Additional criteria for amacrine cell subtype classification include the number of laminae their neurites span in the inner plexiform layer, the neurotransmitters they secrete, and the specific markers they express.
Despite these myriad morphologies, little is known about the factors that determine amacrine cells’ morphological patterning. Previous studies have suggested the importance of extrinsic signals contributing to neural patterning in the retina (Lohmann et al., 2002; Kay et al., 2004; Godinho et al., 2005), drawn from the extensive characterization of amacrine cell morphology in vivo (Kolb et al., 1981; MacNeil and Masland, 1998). Drawing conclusions about any contribution of intrinsic or cell-autonomous contributions to neurite patterning, however, is naturally limited by the lack of ex-vivo studies.

The use of in vitro cultures may help elucidate whether there is any role for intrinsic regulation of neurite growth. For example, purified cultures were previously used to demonstrate developmental regulation of intrinsic axon growth ability in retinal ganglion cells (RGCs) (Goldberg et al., 2002a). Here we take advantage of our ability to highly purify retinal amacrine cells to ask, do purified amacrine cells in vitro recapitulate the neurite grown patterns that they have in vivo? These data argue for at least a contribution of intrinsic neurite growth programs to amacrine cells’ varied neurite morphologies in vivo.

MATERIALS AND METHODS

Amacrine cell and RGC purification

Sprague-Dawley rats were used for these experiments in compliance with institutional animal care and use committee review and approval. Amacrine cells and RGS were purified by immunopanning as previously described (Barres et al., 1988; Meyer-Franke et al., 1995; Goldberg et al., 2002a; Kunzevitzky et al.,
Briefly, retinas were dissected from embryonic day 20 (E20) and postnatal day 8-10 (P8-P10) rats and dissociated with papain (Worthington, Lakewood, NJ) using gentle mechanical trituration to obtain a single-cell suspension. After depleting the retinal suspension of macrophages, RGCs were positively selected using T11d7 monoclonal antibodies (Lake et al., 1979). Enrichment of amacrine cells to ~90% purity was achieved subsequently by depleting Ox7-positive cells (remaining RGCs and other retinal neurons), and immunopanning for Vc1.1-positive cells (Arimatsu et al., 1987).

**Amacrine cell and RGC culture**

Acutely purified amacrine cells and RGCs were cultured at low density on PDL-coated glass coverslips (70 kDa, 10 µg/ml; Sigma), without and with laminin respectively (Trevigen Gaithersburg, MD) in serum-free Neurobasal media (Gibco, Carlsbad, CA) as described (Meyer-Franke et al., 1995; Goldberg et al., 2002a; Kunzevitzky et al., 2009) containing insulin (50 ng/ml; Sigma), forskolin (5 mM; Sigma), CNTF (10 ng/ml; Peprotech, Rocky Hill, NJ), BDNF (50 ng/ml; Peprotech, Rocky Hill, NJ) and a modified version of B27 (Chen et al., 2008). Under these conditions, survival of amacrine cells at 3DIV is ~60% (Kunzevitzky et al., 2009). Cultures were maintained at 37°C in a humidified incubator with 10% CO₂ and either immunostained or processed for neurite growth analysis as described below.
**Immunofluorescence**

For immunostaining of the retina, eyes were dissected from E20 and P9 Sprague-Dawley rats. Corneas and vitreous bodies were carefully removed and the eyecups were immediately fixed with 4% paraformaldehyde for 2 hours, after which the tissues were cryoprotected in 30% sucrose, snap frozen in mounting medium (OCT Tissue-Tek, Electron Microscopy Sciences, Hatfield, PA), and sectioned. Sections were postfixed in 4% paraformaldehyde or 10% trichloroacetic acid (TCA) for 10 min, then blocked and permeabilized with 20% goat or donkey serum and 0.2% Triton X-100 for 30 min. Retinal tissues were incubated overnight with anti-Vc1.1 (1:100; Sigma, St. Louis, MO), anti-HPC-1 (1:200; Abcam, Cambridge, MA), anti-parvalbumin (PV) (1:500; Sigma, St. Louis, MO), anti-calretinin (1:5000), anti-glutamate transporter 1 (GLYT-1) (1:2000) and anti-tyrosine hydroxylase (TH) (1:100; BD Biosciences, Mississauga, ON Canada). Secondary detection was performed using fluorescent antibodies at a 1:500 (Alexa-488, Alexa-594) or a 1:200 dilution (Alexa-647; Invitrogen, Carlsbad, CA). The slides were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and examined with a Zeiss inverted fluorescent microscope or a Leica TCS SP5 confocal microscope.

Immunocytochemistry of purified amacrine cells as in Fig. 2.1 was performed at 2 and at 24 hrs after plating the cells on PDL-coated glass coverslips in serum-free media. Antibodies used are described above.
**Phallloidin staining and neurite growth analysis**

Amacrine cells and retinal ganglion cells were acutely purified from embryonic (E20) and postnatal (P8-P11) animals and plated at clonal density on glass coverslips in serum-free media as described above. After 3 days in vitro (DIV), cells were carefully fixed with warm 4% paraformaldehyde for 25 minutes. Blocking and membrane permeabilization were performed as described above and TRITC-conjugated phallloidin (3.3 µg/ml; Sigma, St. Louis, MO) was added and left overnight at 4°C together with DAPI (Invitrogen, Carlsbad, CA) for nuclear staining. Neurite length measurements were performed at 20X and 63X objective magnification using an inverted fluorescence microscope and Axiovision software (Zeiss, Thornwood, NY); data analysis was conducted using Excel (Microsoft) and SPSS statistical software (SPPS, Chicago, IL.) At least 60 cells were analyzed per experiment. Five different variables were recorded for each cell: number of primary neurites coming off the cell body >=10 µm and their individual length, number of branch points with branches >=10 µm and the neurite length of each branch, and length of the longest neurite without its branches. Combination of these values allowed for the calculation of the rest of the parameters used in data analysis: total neurite length (sum of the length of all primary neurites and their branches); number of segments (number of primary neurites + 2 x number of branch points); and average segment length (total neurite length divided by the number of segments). Comparison of neurite growth variables by cell type yielded significantly high variances between cell types for
all variables; therefore, ANOVA overall group comparison were verified with the Kruskal Wallis test, followed by Tamhane’s multiple comparison procedure.

**Neurite growth analysis of amacrine cell subpopulations.**

Postnatal day 8 amacrine cells were purified and plated on PDL-coated coverslips as described above. At 3 DIV, cells were carefully fixed in warm 4%PFA and processed for immunostaining and phalloidin labeling as previously described. Cells were manually traced as indicated in the phalloidin (neurites) channel to mask the observer, and grouping of cells by immunoreactivity to amacrine cell subtype markers was assessed later on. Measurements of immunoreactive cells were compared against their negative counterparts within the same group. At least 50 immunoreactive cells were analyzed per group and the means were compared using unpaired Student’s t-test.

**RESULTS**

**Purified amacrine cell subpopulations during development**

We purified E20 and P9 amacrine cells by immunopanning and asked whether these amacrine cell subpopulations change greatly through the early embryonic to late postnatal period. We immunostained acutely purified cells at either two hours after plating or at 1 day in vitro (DIV), and found that, at 2 hours in vitro, fewer E19 amacrine cells were immunoreactive to GLYT-1 (Fig. 2.1C, D), PV (Fig. 2.1E, F) and TH (trend level only; Fig. 2.1G, H) than P9 amacrine cells, whereas calretinin immunoreactivity was statistically similar at the two ages (Fig.
2.1A, B). After 1 DIV, E19 amacrine cells conserved their immunoreactivity to TH and Vc1.1 (Fig 2.1H; Suppl. Fig 2.1) but decreased in immunoreactivity to GLYT-1 and PV (Fig. 2.1D, F), due to either in vitro aging or a relative decrease in survival of these cells at this particular developmental age. We found no significant changes in immunoreactivity of P9 amacrine cells from 2 hours to 1 DIV (Fig. 2.1B, D, F), except for an increase in TH-immunoreactive (TH-IR) cells (Fig. 2.1H). Immunostaining of retinal cross-sections showed similar differences in amacrine cell subpopulation immunoreactivity for calretinin, GLYT-1, PV and TH between E19 and P8 (Fig. 2.1). Taken together, these data suggest that significant amacrine cell differentiation, at least in the expression of this subset of subpopulation markers, continues through this developmental period, consistent with prior literature on the coordinated expression of neurotransmitters at all stages during retinal development (Kong et al., 1980; Lam et al., 1981; Fung et al., 1982; Parkinson and Rando, 1984; Redburn and Rowe-Rendleman, 1996). Importantly, we found that these subtype marker phenotypes are expressed in mostly stable proportions in vitro.

**Amacrine cell neurite extension in vitro**

Amacrine cells extend complex and varied neurites in vivo; the variation in their neurite patterning has been used to characterize them in vivo into subtypes based on morphology (MacNeil and Masland, 1998; MacNeil et al., 1999; Lin and Masland, 2006). We asked whether this varied morphology is recapitulated in vitro. We purified embryonic and postnatal amacrine cells and cultured them in
defined, serum-free media. At 3 DIV, amacrine cells were stained with phalloidin to visualize neurite morphology and their neurites were manually traced (Fig. 2.2A). We collected data on 5 different parameters of neurite growth: the number of primary neurites extending from the cell body; the number of branch points; the length of each primary neurite with its branches; the length of every branch; and the length of the longest neurite without its branches. We used these data to calculate 3 additional variables: the total neurite length, the number of segments (defined as a stretch of neurite bounded by the cell body, a branch point, or the end of a neurite), and the average segment length (see Methods and Fig. 2.2A).

We analyzed these different neurite growth parameters and found that postnatal amacrine cells could extend neurites as long as 700 \( \mu \text{m} \), although the majority of them (60\%) extended neurites less than 150 \( \mu \text{m} \) long (Fig. 2.2B). Consistent with the existence of axon-bearing amacrine cells (Dacey, 1989, 1990), in our cultures we found that \(~40\%\) of the postnatal amacrine cells extended one lengthy process, typically 20-40 \( \mu \text{m} \) long (Fig. 2.2C).

Embryonic amacrine cells also grew one lengthier neurite of similar size on average (47 \( \mu \text{m} \)). Comparing this phenomenon with RGCs, we found that postnatal RGCs, on average, have a longest neurite (axon) 4 times larger (195 \( \mu \text{m} \)) and that in embryonic RGCs the longest neurite is almost 10 times as long (430 \( \mu \text{m} \); Table 2.1). Thus embryonic and postnatal amacrine cells extend neurites of similar lengths, unlike RGCs, which extend considerably longer axons in the embryonic period than in the postnatal period (Goldberg et al., 2002a).
Quantifying the number of neurites per cell and the average segment length roughly grouped the amacrine cells into clusters (Fig. 2.2D, E) suggesting the existence of more than one cell subpopulation in our cultures. To address this observation, and ask whether in vitro amacrine cells retain morphologic diversity in vitro analogous to that exhibited in vivo, we performed a principal component analysis. This is a multivariate analysis method that reduces our original 7 variables into fewer components when (and if) these variables are highly correlated. We found that 3 components were able to explain 90 to 99% of the variance in the samples analyzed (Table 2.2). Component 1, which accounted for approximately 53% of the variability in amacrine cell neurite growth, described a variable largely based on total neurite growth capacity, with a spectrum running from short, simple cells on one end to long, complex neurite morphologies on the other (example cells in Fig. 2.3). Component 2, which explained 24% of the variability in amacrine cell neurite growth, ran from short, highly branched neurites on one end to long neurites with fewer branches on the other (Fig. 2.3). This suggests that a significant component of neurite growth is explained by a mechanism that trades branching for total length. Finally, component 3 accounted for 18% of the variability, and displayed a wide spectrum of neurite shape and length complexity, where large numbers of neurites and longer lengths were on one end and large numbers of branches were on the other end (Fig 2.3). Interestingly, the principal component analysis yielded remarkably similar data for embryonic and postnatal amacrine cells and postnatal RGCs (Table 2.2), but embryonic RGCs differed slightly in that they had the longest
neurite length per neuron and a corresponding larger average segment length (Table 2.1), and their “Component 2,” trading length for branching, was extracted first and explained the most variance (29%) rather than the second most, as in all of the other populations studied (Table 2.2). Thus, these data suggest that “component 2” trading branching versus length is more important for characterizing E20 RGCs than “component 1” overall size, whereas for postnatal RGCs and amacrine cells at all ages, overall size is a more important feature of neurite growth.

**Neurite outgrowth of amacrine cell subpopulations**

Extensive evidence suggests that extrinsic, environmental signals are critical for shaping amacrine cell morphologies *in vivo*, but there is less evidence to suggest whether the variety of morphologies *in vivo* also depends on an intrinsic or cell-autonomous component. To address this question, we asked whether purified amacrine cells would retain any intrinsic neurite growth properties typical of their *in vivo* growth patterns. We purified amacrine cells, characterized them by immunocytochemistry and measured their neurite outgrowth as described above (see Methods).

We first asked whether parvalbumin-immunoreactive (PV-IR) amacrine cells in vitro replicate their in vivo morphologies. Previous camera lucida reconstructions of PV-IR cells of the rabbit retina show cells with a pyriform cell body and cell processes with lobular appendages in one sublamina and arboreal processes in another sublamina (Casini et al., 1995) (Fig. 2.4A), consistent with
the description of all amacrine cells revealed by the use of intracellular labels such as Golgi stain (Famiglietti and Kolb, 1975), horseradish peroxidase and Lucifer Yellow (Nelson, 1982). We found that parvalbumin-immunoreactive (PV-IR) cells trended towards a lower number of segments, number of primary neurites coming off the cell body, number of branch points, longest neurite and total neurite length compared to non-PV-IR amacrine cells (Fig. 2.4B-C). However, because of the high variability of these values (Suppl. Table 2.1), the difference between the means was not statistically significant. PV-IR cells did demonstrate lobular varicosities on their neurites in vitro similar to those seen in vivo (arrows in Fig. 2.4B). Thus PV-IR amacrine cells in vitro demonstrate a morphology similar to that in vivo (Fig. 2.4B).

We next investigated the morphology of TH-IR cells in vitro. These cells are part of the family of wide field amacrine cells and in vivo, they have been described as cells with long (hundreds of micrometers), thin, unbranched, axon-like processes with few spines or varicosities (Brecha et al., 1984; Dacey, 1990; Mariani, 1990; Casini and Brecha, 1992; MacNeil et al., 1999) (Fig. 2.5A). Consistent with their morphology in vivo, TH-IR amacrine cells extended fewer primary neurites in vitro that were 33% longer on average than those of non-TH-IR amacrine cells. Overall, the total neurite length of TH-IR cells was 50% larger than in non-TH-IR amacrine cells (Fig. 2.5B, C). Thus, for TH-IR cells, there was striking morphological similarity among purified amacrine cells and the cells described in the literature in the mammalian retina (Fig. 2.5A) (Brecha et al., 1984; Casini and Brecha, 1992; MacNeil et al., 1999).
Finally, we examined the morphology of glycinergic amacrine cells in vitro and found that they too resembled the shape of glycinergic amacrine cells in vivo (Fig. 2.6A, B) (Menger et al., 1998). Glycinergic amacrine cells, widely known as AII amacrine cells, are characterized by their narrow field and a bushy dendritic tree coming off a single neurite that can span many sublaminae in the IPL (Fig. 2.6A). In our cultures, cells that were immunoreactive to the glycinergic transporter 1 (GLYT-1-IR cells) had a significantly greater number of segments and a total neurite length ~2-fold larger than controls (Fig. 2.6C). The number of primary neurites coming off the cell body was significantly higher in GLYT-1-IR cells (Fig. 2.6B, C). Taken together, these data show that in vitro, amacrine cells elaborate neurites with a variety of complexities reminiscent of the variation in patterning seen in vivo, and support the hypothesis that at least a portion of their patterning variability in vivo may depend on cell-intrinsic mechanisms.

**DISCUSSION**

Understanding the molecular and cellular basis for the morphological heterogeneity of neurons in the central nervous system remains a major goal in neuroscience. Aamacrine cells in the mammalian retina represent an excellent model system in which to study this question, as they demonstrate remarkable morphologic heterogeneity (Kolb et al., 1981; Nelson et al., 1981; Jeon et al., 1998; Masland, 2001). Although the variation in amacrine cell morphology has been carefully characterized in vivo, little work has focused on their properties in vitro. Similar work on other populations of CNS neurons has yielded fruitful
observations about neurite growth properties; for example, the signals optimal for survival and neurite growth of RGCs have been characterized using such cultures (Goldberg and Barres, 2000; Goldberg et al., 2002b). Here we took advantage of our ability to highly purify these neurons by immunopanning to study their neurite growth away from neuronal- or glial-derived signals found in the in vivo environment. We cultured purified amacrine cells and measured several neurite growth parameters, including total neurite length and the number of primary neurites and branch points. In vitro, a number of amacrine cell subpopulations extended neurites of varied lengths and morphologies similarly as they do in vivo, raising the hypothesis that the regulation of these processes has an intrinsic component.

**Total neurite length conservation in amacrine cell neurite growth**

Detailed analysis of neurite morphology in vivo has suggested that at least some neurons maintain a constant total neurite length when they grow neurites, trading off between neurite length and branching (Samsonovich and Ascoli, 2006). Our data using principal component analysis suggest that the second greatest component that captures the variance in amacrine cell neurite growth follows this principle of trading neurite length for complexity (branching), and supports the hypothesis that the biology that underlies this observed conservation may be cell-autonomous. The increased importance of this conservation principle in embryonic RGCs (Table 2.2) may ultimately explain their dramatically increased axon growth ability compared with either amacrine
cells, or with postnatal or adult RGCs (Goldberg et al., 2002a). The underlying biology could involve limitations on supply of any single or number of building blocks for neurite elongation, for example cytoplasmic or membrane components (Goldberg, 2003), or a feedback between the cell body and neurites or growth cones, but remains to be discovered.

**Intrinsic regulation of amacrine cell neurite growth**

It is not known whether the varied morphology of amacrine cell neurites in vivo (Kolb et al., 1981; MacNeil et al., 1999; Masland, 2001) is attributable to cell intrinsic or extrinsic signals, or both. Evidence for the role of extrinsic cues in neurite patterning in the retina came from experiments addressing the role of activity in RGCs’ dendrite remodeling during development. In these experiments, a decrease in local calcium concentration at the tip of the dendrites was sufficient to alter the dendritic organization of RGCs (Lohmann et al., 2002). Further evidence for the importance of extrinsic cues in neurite patterning was demonstrated in mutant zebrafish, where in the absence of RGCs, amacrine cells failed to properly direct their projections to the IPL and the sublaminar architecture of the retina was temporarily disorganized (Kay et al., 2004). Over time, most of the projection errors were corrected and the sublaminae were formed, suggesting that the IPL contained other cues, including possible the amacrine cell neurites themselves, that were important for lamination (Godinho et al., 2005).
Here we find that a number of amacrine cell subtypes recapitulate aspects of their in vivo morphology in vitro. Consistent with prior characterizations of amacrine cell subpopulations in the mammalian retina (Kolb et al., 1981; Brecha et al., 1984; Pourcho and Goebel, 1985; Mariani and Hokoc, 1988; Kolb et al., 1990; Wassle et al., 1993; Gustincich et al., 1997; Menger et al., 1998), in our cultures, PV-IR cells grew processes with lobular appendages and multiple varicosities (Casini et al., 1995; Casini et al., 1998). TH-IR cells had long axon-like, unbranched processes (Dacey, 1990) and GLYT-1-IR cells also exhibited a typical bushy neurite morphology (Menger et al., 1998), although our quantitative analysis of number of branch points and branching index of GLYT-1-IR cells did not yield statistically significant differences with the non-IR cells, possibly because our analyses did not include branches <10 μm. Nevertheless, these data suggest that amacrine cells have cell subtype-specific intrinsic determinants of neurite growth that are carried into culture.

Despite the strong evidence for the role of extrinsic cues in determining neurite patterning in the developing retina, a few prior results support the hypothesis of intrinsic regulation. For example, during the initial phase of lamination in the developing zebrafish retina, amacrine cells extend processes indiscriminately that are not directed towards their final destination, the IPL (Godinho et al., 2005). This random extension of processes may represent a brief developmental period of neurite growth relatively free of guidance from extrinsic cues. The recent creation of transgenic mouse lines that express fluorescent protein reporters under the control of amacrine cell-specific
promoters (for example, as in Haverkamp et al., 2009) will certainly allow these questions to be further addressed. Taken together, these data support the existence of genetically programmed, intrinsic regulation of amacrine cells’ neurite growth and patterning in the developing retina.
CHAPTER FIGURES
Figure 2.1 Immunostaining of amacrine cells in vitro and in vivo. (A,C,E,G) Embryonic day 19 (E19) or postnatal day 8 (P8) amacrine cells were purified, plated in serum-free media and immunostained as labeled. E19 and P8 retinal cross-sections were stained for the same amacrine cell markers. (B,D,F,H) Quantification of immunoreactivity of amacrine cells in vitro at 2 hours (hIV) or 1 day (DIV) after plating for each marker as labeled. (#p=0.055 “trend”; *p<0.05; **p<0.01; Student’s t-test.)
Figure 2.2 Different populations of amacrine cells can be identified by their polarization patterns in vitro. (A) Postnatal day 9 amacrine cells were purified by immunopanning and cultured in the presence of trophic factors. After 3 days in vitro, the cells were fixed and stained with TRITC-conjugated phalloidin. Images were analyzed in a fluorescence microscope and the neurites were manually traced using Axiovision software (Zeiss). Scale bar, 20 µm. (B-E) Postnatal amacrine cells and RGCs were purified and plated for 3 days, after which cells were stained with phalloidin and their neurites manually traced (E20 RGCs, n=62; E20 amacrine, n=73; postnatal RGCs, n=218; postnatal amacrine cells, n=323).
**Figure 2.3** Amacrine cell morphology *in vitro* and contribution to principal component analysis. Example images of postnatal amacrine cells at the extremes of the principal component axes described in Table 2.2 are shown here. Scale varies slightly to demonstrate neurite growth in each picture.
Figure 2.4 Parvalbumin immunoreactive amacrine cells *in vivo* and *in vitro*. (A) Camera lucida reconstructions of parvalbumin immunoreactive cells from rabbit inferior retina (image reproduced from Casini et al., 1995). (B) Purified amacrine cells immunoreactive to parvalbumin (yellow) and counterstained with TRITC-conjugated phalloidin show a similar morphology *in vitro*. The arrows show examples of lobular processes. (C) Quantification of neurite growth parameters of PV-IR amacrine cells *at 3DIV*. The bars represent the values of PV-IR cells (*n*=70 cells) normalized to non-IR cells within the experiment (*n*=53 cells). Error bars: SEM of the PV-IR cells.
Figure 2.5 Tyrosine hydroxylase immunoreactive amacrine cells *in vivo* and *in vitro*. (A) Camera lucida reconstructions of tyrosine hydroxylase immunoreactive cells in the ventral part of adult rabbit retina (image reproduced from Casini et al., 1992); scale bar = 50 μm. (B) Purified amacrine cells immunoreactive to tyrosine hydroxylase (yellow) and counterstained with TRITC-conjugated phalloidin show a similar morphology *in vitro*. (C) Quantification of neurite growth parameters of TH-IR amacrine cells at 3DIV. The bars represent the values of TH-IR cells (n=88 cells) normalized to non-IR cells within the experiment (n=56 cells); *p< 0.05, unpaired Student’s t-test, error bars: SEM of the TH-IR cells.
Figure 2.6 GLYT-1 immunoreactive amacrine cells in vivo and in vitro. (A) Illustrations of 8 types of GLYT-IR cells from rat retina spanning different sublaminae of the IPL (image reproduced from Menger et al, 1995). (B) Purified amacrine cells immunoreactive to GLYT-1 (yellow) and counterstained with TRITC-conjugated phalloidin show a similar morphology in vitro. (C) Quantification of neurite growth parameters of GLYT-1-IR amacrine cells at 3DIV. The bars represent the values of GLYT-1-IR cells (n=67 cells) normalized to non-IR cells within the experiment (n=67 cells); *p<0.05, **p<0.01, ***p<0.001, unpaired Student’s t-test; error bars: SEM of the GLYT-1-IR cells.
Supplemental figure 2.1 Amacrine cell immunostaining with Vc1.1 antibody. Acutely purified amacrine cells were plated in serum-free media and immunostained at 2 hours or at 1DIV. The bars show percentage of cells immunopositive for Vc1.1 of the total number of cells labeled with the nuclear dye DAPI. (Error bars: SEM.)
## CHAPTER TABLES

**Table 2.1** Comparison of neurite growth variables in amacrine cells and RGCs.

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</tr>
<tr>
<td><strong>Total neurite length</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>184.35</td>
<td>151.38</td>
<td>643.16</td>
<td>642.90</td>
<td>370.90</td>
<td>&lt;0.001</td>
<td>RGCs P8, RGCs E20 &gt; Amacrines P8-P10, Amacrines E20</td>
</tr>
<tr>
<td>SD</td>
<td>173.26</td>
<td>151.11</td>
<td>323.97</td>
<td>287.36</td>
<td>332.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>121.77</td>
<td>123.35</td>
<td>615.19</td>
<td>631.16</td>
<td>266.31</td>
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<tr>
<td>Minimum</td>
<td>10.35</td>
<td>10.93</td>
<td>18.29</td>
<td>76.00</td>
<td>10.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum</td>
<td>861.32</td>
<td>1119.10</td>
<td>2673.63</td>
<td>1778.01</td>
<td>2073.63</td>
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</tbody>
</table>

Since variances are significantly different between cell types for all variables, ANOVA overall group comparison were verified with the Kruskal Wallis test, followed by Tamhane’s multiple comparison procedure.
Table 2.2 Principal components analysis.

<table>
<thead>
<tr>
<th>Principal Component Analysis</th>
<th>P6-P10 Amacrine PC 1</th>
<th>P6-P10 Amacrine PC 2</th>
<th>P6-P10 Amacrine PC 3</th>
<th>E20 Amacrine PC 1</th>
<th>E20 Amacrine PC 2</th>
<th>E20 Amacrine PC 3</th>
<th>P8 RGCs PC 1</th>
<th>P8 RGCs PC 2</th>
<th>P8 RGCs PC 3</th>
<th>E20 RGCs PC 1</th>
<th>E20 RGCs PC 2</th>
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<tbody>
<tr>
<td>Number of Segments</td>
<td>0.21</td>
<td>-0.23</td>
<td>-0.13</td>
<td>0.15</td>
<td>-0.28</td>
<td>-0.16</td>
<td>0.28</td>
<td>-0.22</td>
<td>0.04</td>
<td>0.25</td>
<td>-0.22</td>
<td>-0.14</td>
</tr>
<tr>
<td>Number of Neurites &gt;10μm</td>
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<td>-0.12</td>
<td>-0.47</td>
<td>0.10</td>
<td>-0.17</td>
<td>-0.81</td>
<td>0.00</td>
<td>-0.44</td>
<td>-0.18</td>
<td>0.03</td>
<td>-0.17</td>
<td>-0.48</td>
</tr>
<tr>
<td>Number of Branchpoints &gt;10μm</td>
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<td>-0.24</td>
<td>0.12</td>
<td>0.14</td>
<td>-0.25</td>
<td>0.38</td>
<td>0.27</td>
<td>-0.13</td>
<td>0.08</td>
<td>0.32</td>
<td>-0.16</td>
<td>0.28</td>
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<tr>
<td>Branching Index</td>
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<td>0.53</td>
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<td>0.44</td>
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<td>Average Segment Length</td>
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<td>-0.45</td>
<td>0.07</td>
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<td>0.03</td>
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<tr>
<td>Average Neurite Length</td>
<td>0.17</td>
<td>0.22</td>
<td>0.36</td>
<td>0.22</td>
<td>0.16</td>
<td>0.13</td>
<td>0.20</td>
<td>0.36</td>
<td>-0.02</td>
<td>0.17</td>
<td>0.24</td>
<td>0.16</td>
</tr>
<tr>
<td>Longest Neurite Length</td>
<td>0.17</td>
<td>0.31</td>
<td>-0.08</td>
<td>0.21</td>
<td>0.19</td>
<td>0.04</td>
<td>0.08</td>
<td>0.12</td>
<td>-0.45</td>
<td>0.24</td>
<td>0.19</td>
<td>-0.23</td>
</tr>
<tr>
<td>Total Neurite Length</td>
<td>0.22</td>
<td>0.04</td>
<td>-0.18</td>
<td>0.24</td>
<td>0.04</td>
<td>-0.18</td>
<td>0.22</td>
<td>-0.14</td>
<td>-0.30</td>
<td>0.32</td>
<td>0.16</td>
<td>-0.35</td>
</tr>
<tr>
<td>Variance Explained</td>
<td>53%</td>
<td>24%</td>
<td>18%</td>
<td>50%</td>
<td>37%</td>
<td>12%</td>
<td>42%</td>
<td>25%</td>
<td>23%</td>
<td>28%</td>
<td>44%</td>
<td>19%</td>
</tr>
</tbody>
</table>

Negative component coefficients (yellow) for a variable indicate that cells with large values for that observed variable tend to sort towards one end of that underlying variable, while positive coefficients (blue) indicate they sort towards the other end of that underlying variable. Coefficients with absolute value < 0.1 indicate that the observed variable does not contribute much to the underlying principal component.
CHAPTER THREE: Foxn4 Is Required for Normal Retinal Ganglion Cell Axon Projection to the Superior Colliculus

CHAPTER OVERVIEW

The regulation of retinal ganglion cell (RGC) axon growth and patterning in vivo is thought to be largely dependent on interactions with visual pathway and target cells. However, evidence for presynaptic regulation of RGC functions comes from our previous demonstrations that RGCs undergo an irreversible loss of intrinsic axon growth ability during development that can be signaled by amacrine cells, raising the hypothesis that these presynaptic neurons may regulate RGC axon growth or targeting. Here we asked whether the normal development of amacrine cells is required for proper RGC axon growth in vivo. We found that in Foxn4−/− mice, which have fewer amacrine cells, there is a developmental delay in the distribution of RGC projections to the superior colliculus. Furthermore, RGC axons fail to penetrate into the retinorecipient layers. These findings suggest that amacrine cells are critical for proper RGC axon growth in vivo, and raise the hypothesis that the amacrine cell-RGC interaction may regulate distal projections and axon patterning.

CHAPTER INTRODUCTION

How is the axon growth, guidance and patterning of projection neurons regulated? For example, retinal ganglion cells (RGCs) extend their axons into the nerve fiber layer of the retina, grow towards the optic nerve head attracted to netrin-1 (Holt, 1989; Serafini et al., 1994; Serafini et al., 1996; Leonardo et al.,
1997) and sonic hedgehog (Neumann and Nuesslein-Volhard, 2000; Kolpak et al., 2005), are channeled down the optic nerve by semaphorin-5A (Oster et al., 2003), either cross or remain ipsilateral at the optic chiasm in response to heparan and chondroitin sulfate proteoglycans (CSPGs) (Brittis et al., 1992; Brittis and Silver, 1995; Kantor et al., 2004), and pattern their gross connections at the superior colliculus in response to ephrins (Frisen et al., 1998; Nakagawa et al., 2000; Huber et al., 2003; McLaughlin and O'Leary, 2005), Slits (Nicolou et al., 2000; Thompson et al., 2006a; Thompson et al., 2006b) and CSPGs (Chung et al., 2000). Although these are clearly the properties of ligands and adhesion molecules secreted by intermediate guidepost and target cells, intrinsic properties of RGCs also determine axon targeting. For example, the RGC transcription factor Brn3b is required for normal axon guidance and mapping in the superior colliculus (Erkman et al., 2000; Badea et al., 2009) and the transcription factor Zic2 dictates which RGCs will remain ipsilateral at the optic chiasm (Herrera et al., 2003).

It is not well understood whether presynaptic signals also regulate axon growth for proper wiring to occur. We previously showed that embryonic RGCs undergo a developmental loss of their intrinsic capacity for rapid axon growth (Goldberg et al., 2002a), which could likewise contribute to an intrinsic regenerative failure in adult CNS neurons (Chen et al., 1995; Dusart et al., 1997; Gao et al., 2003; Chierzi et al., 2005). Interestingly, we found that RGCs' developmental loss of axon growth ability could be signaled by their presynaptic
retinal partners, amacrine cells. This raises the question, do amacrine cells regulate RGC axon growth in vivo?

Recently it was demonstrated that in the mammalian retina, Foxn4 controls amacrine cell fate specification from retinal progenitors, and that Foxn4<sup>−/−</sup> mice had significantly fewer amacrine cells than their wildtype littermates (Li et al., 2004). These mice also lacked horizontal cells, whereas the differentiation of other cell types in the retina did not seem to be altered. It is not known whether amacrine cell-RGC signaling may control RGC axon growth during normal development. Because amacrine cells can signal RGCs <i>in vitro</i> to decrease their intrinsic axon growth ability (Goldberg et al., 2002a), we hypothesized that in the developmental absence of amacrine cells, RGCs might retain their embryonic axon growth ability, or perhaps project their axons abnormally. Here we show that Foxn4 is required for proper outgrowth of RGC axons in vivo, suggesting a role for amacrine cells-RGCs interaction in axon growth.

**MATERIALS AND METHODS**

Animal experiments were conducted in accordance with the guidelines of the University of Miami Institutional Animal Care and Use Committee (IACUC) and comply with the ARVO Statement for the Use of Animals in Research.

**Foxn4<sup>−/−</sup> mice**

Foxn4<sup>+/−</sup> females were obtained from the Xiang laboratory (Li et al., 2004) and bred to C57/Bl6 males; heterozygotes were interbred to generate knockout
mice with heterozygote and wildtype littermates. Mice were genotyped by PCR using genomic DNA from clipped tails following standard protocols. Specific primer sequences for Foxn4 and LacZ were: Foxn4: 5'-GGCCTCTCTGTCCATACCTGTA-3' (forward) and 5'-CTACTCTCTTTGATGACAGCTCCC-3' (reverse); LacZ: 5'-GGTTGTTACTCGCTCACATTATAATG-3' (forward) and 5'-CCATGCAGAGGATGATGCTCGTGAC-3' (reverse). The PCR product of wildtype mouse DNA consisted of a single band of 460 bp (Foxn4 only); amplification of heterozygous and knockout mouse DNA yielded either two bands of 460 bp (Foxn4) and 730 bp (LacZ) or a single band of 730 bp (LacZ only), respectively.

Immunofluorescence

For immunostaining of retina, animals were perfused and eyeballs were collected and fixed with 4% paraformaldehyde (PFA) for 1 hour, after which the tissues were cryoprotected overnight in 30% sucrose, snap frozen in mounting medium (OCT Tissue-Tek, Electron Microscopy Sciences, Hatfield, PA), and sectioned. Sections were postfixed in 4% paraformaldehyde and 10% trichloroacetic acid (TCA) for 10 minutes, then permeabilized with 0.2% Triton X-100 for 30 minutes, and further blocked and permeabilized with 20% normal goat or donkey serum and 0.2% Triton X-100 for 1 hour. Retinal tissues were incubated overnight with anti-Vc1.1 (1:100; Sigma, St. Louis, MO), anti-HPC-1 (1:200; Abcam, Cambridge, MA), anti-GAD65/67 (1:1000), anti-parvalbumin
(1:500; Sigma, St. Louis, MO), anti-calretinin (1:5000), anti-glutamate transporter 1 (1:2000), anti-tyrosine hydroxylase (1:100; BD Biosciences, Mississauga, ON Canada), and anti-Map2 (1:150, Sigma, St. Louis, MO). Secondary detection was performed using fluorescent antibodies at a 1:500 (Alexa-488, Alexa-594) or a 1:200 dilution (Alexa-647; Invitrogen, Carlsbad, CA). Slides were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and examined in a Zeiss inverted fluorescent microscope or a Leica TCS SP5 confocal microscope.

Immunocytochemistry of purified retinal ganglion cells was performed as previously described (Wang et al., 2007). Briefly, cells were fixed with 4% PFA for 10 minutes, rinsed three times in PBS, and blocked and permeabilized for 30 minutes with 20% normal goat serum and 0.2% Triton X-100 in antibody buffer (150mM NaCl, 50mM Tris base, 1% BSA, 100mM L-Lysine, 0.04% Na azide, pH 7.4). Overnight incubation with rabbit anti-Tau (1:400, Sigma-Aldrich, St Louis, MO) was performed at 4°C. Goat anti-rabbit Alexa 647 was used at a 1:200 dilution for secondary detection and DAPI was added for nuclear staining. Cells were rinsed and kept in PBS for imaging. (See below.)

Immunofluorescence of brain tissues with Foxn4 antibodies was performed as previously described (Li et al., 2004). Briefly, P3 mice were perfused and euthanized in compliance with the University of Medicine and Dentistry of New Jersey IACUC, after which the brains were dissected and fixed for 2 hours in 4% PFA in PBS at 4°C. Following 30% sucrose infiltration and embedding in OCT (Tissue-Tek, Electron Microscopy Sciences, Hatfield, PA), the samples were sectioned and the cryosections were used for
immunofluorescence. Foxn4 antibody was applied at 1: 50 dilution following overnight incubation as previous reported. Images of these sections were obtained with a Nikon eclipse 80i microscope.

**Imaging of retinal waves**

Freshly dissected retinas from Foxn4−/− mice and their wild-type littermates between the day of birth (P0) and postnatal day 5 (P5) were wicked onto filter paper with the ganglion cell layer facing up and incubated in a solution of 10 µM Fura 2-AM (Invitrogen, Carlsbad, CA) in artificial CSF (ACSF) containing 1% DMSO and 0.02% pluronic acid (Invitrogen, Carlsbad, CA) for 60 to 90 minutes in an oxygenated glass chamber at 33°C. After rinsing with ACSF, retinas were kept in a temperature-controlled chamber at 33°C, mounted on the stage of an inverted microscope (Axiovert 200M, Zeiss) and cultured in ACSF (119 mM NaCl, 2.5 mM KCl, 1.3 mM MgCl₂, 1.0 mM KH₂PO₄, 2.5 mM CaCl₂, 26.2 mM NaHCO₃, and 11 mM D-glucose) in a controlled environment (95% O₂ / 5% CO₂). Retinas were illuminated at 340 and 380 nm excitation wavelength with a 175 Watt, ozone-free xenon lamp (Lambda DG-4, Nikon, Japan) and fluorescence intensities were detected at 500 nm. Images were collected at 5 Hz under 20x objective lens (NA=0.4) with a cooled CCD camera (Coolsnap ES Monochrome, Photometrics). Initial single images were collected to identify neurons in the ganglion cell layer including retrograde labeled RGCs where relevant. After data acquisition, areas of interests were selected and the files were exported to the MetaFluor software (Universal Imaging, CA, USA). Fractional changes in
emission intensity, $\Delta F/F$ defined by the ratio of fluorescence intensities produced by excitation at two wavelengths, were measured from individual cell bodies and automatically exported into Excel software and also displayed as traces of $\Delta F/F$ versus time. Upward deflection indicates the increase of intracellular calcium level, induced by the propagating retinal waves. Only intensity increases above 0.003 and synchronized with others were scored as spikes. Data are expressed as means ± SD unless noted otherwise. Statistical analysis: Student’s t-test, $p<0.05$. Under most circumstances, four areas per retina were selected for imaging.

**Anterograde and retrograde labeling**

Animals were deeply anesthetized by hypothermia. Injection of 1 µl per eye of 1% 488- or 594-cholera toxin subunit B (CTB; Molecular Probes, Invitrogen, Carlsbad, CA) was performed intravitreously using a Hamilton syringe. Different colors were used for each eye, typically 594/red in the right eye and 488/green in the left eye. Six hours later, animals were euthanized and the brains dissected, cutting the optic nerve close to the eyeballs to preserve the optic chiasm. Tissues were immediately fixed in 4% PFA for 2 hours at room temperature, left on a 30% sucrose solution overnight at 4°C and frozen in OCT medium for cryosectioning. 8 µm sections were mounted on glass slides with Vectashield and DAPI (Vector Laboratories, Burlingame, CA) and examined in a Zeiss inverted fluorescent microscope or a Leica TCS SP5 confocal microscope.
Retrograde labeling was performed on postnatal day 1 (P1) pups. 1 µl of a 1% solution of Alexa-594 (red)-conjugated CTB (Molecular Probes) was injected per animal (0.5 µl per side) at 1 mm from bregma and 1 mm from the midline at a depth of 1 mm. Six days later, the procedure was repeated using Alexa-488 (green)-conjugated CTB and pups were euthanized the next day (at postnatal day 8). Eyeballs, optic nerves and optic chiasms were collected and immediately fixed in 4% PFA and processed for cryosectioning as described above.

**RGC purification, cell culture and neurite growth analysis**

RGCs from P2 and P4 mice were purified by immunopanning as previously described (Meyer-Franke et al., 1995; Goldberg et al., 2002a; Moore et al., 2009). Briefly, embryonic and postnatal rat retinas were dissociated with papain (Worthington, Lakewood, NJ) and mechanically triturated to obtain a single cell suspension. Enrichment of RGCs was achieved after sequentially depleting mouse macrophages and selecting CD90 positive cells using anti-Thy1.2 antibody (1:125, AbD Serotec, Oxfordshire, United Kingdom). Because Foxn4−/− mice die soon after birth (Williams et al., 2004; Fujitani et al., 2006) and they do not breed normally, the number of KO animals available in a litter to purify RGCs was limited. We overcame this difficulty by labeling the RGCs using different fluorophores for KO and WT or HET mice. The day before the experiment and after genotyping, we performed intravitreous injections of CTB. The next day, dye-labeled animals were sacrificed and RGCs purified as described above, mixing the KO, WT and HET mouse-derived RGCs for the
purification and plating but still able to differentiate them by color (Fig. 3.5A). Acutely purified RGCs were plated at clonal densities on plated pre-coated with PDL and mouse laminin (Sigma Aldrich, St. Louis, MO). At 2 days in vitro (DIV), cells were fixed in 4% PFA, processed for anti-tau immunofluorescence as described above, and automated neurite tracing was performed at 10X magnification in a KineticScan® HCS Reader (Cellomics, Pittsburgh, PA). The cells from KO and WT mice were identified based on their different fluorescent labels. Data analysis was conducted in Excel (Microsoft). At least 70 cells were analyzed per condition, and the experiments were repeated at least twice.

**Preparation of tissues for electron microscopy**

Optic nerves from postnatal day 4 Foxn4\(^{-/-}\) and wildtype animals were collected and processed immediately for electron microscopy as previously described (Pearse et al., 2004) at the University of Miami Electron Microscopy Core facility. Sections were examined in a Philips CM10 electron microscope. Electron micrographs were taken from cross-sections of at least two animals per genotype.

**RESULTS**

**Decreased amacrine cell number in Foxn4 \(^{-/-}\) mice**

Targeted knockout of Foxn4 leads to a specific loss of amacrine cells and horizontal cells in the mouse retina without affecting the number of retinal
ganglion cells (RGCs) (Li et al., 2004). We stained retinal cross-sections with the nuclear label DAPI and counted the number nuclei in the ganglion cell layer (GCL) and the inner nuclear layer (INL) laying along an arbitrary horizontal line. We confirmed that Foxn4−/− mice have fewer nuclei in the INL and GCL, where amacrine cells reside, compared to their wild-type littermates (Fig. 3.1A, B, E). When we immunostained for two pan-amacrine cell markers, syntaxin and Vc1.1 (Barnstable et al., 1985; Arimatsu et al., 1987), we again found a greatly reduced staining in the inner aspect of the INL and the GCL (Fig. 3.1E), consistent with the prior characterization (Li et al., 2004). Heterozygotes were indistinguishable from wildtypes (data not shown). We also observed decreased Vc1.1 and syntaxin staining of amacrine cell neurites in the inner plexiform layer (IPL) (Fig. 3.1E).

The number of nuclei in the INL and the IPL thickness were similarly decreased in both peripheral and central retina (Fig. 3.1B, C), although a comparison of the nuclei in the INL in the central retina did not reach statistical significance (Fig. 3.1B). To confirm that the effect of the Foxn4 targeted disruption does not affect other layers of the retina, we counted the number of nuclei in the outer nuclear layer (ONL) and found that, as previously described (Li et al., 2004), cells in the ONL remain unaffected (Fig. 3.1D). When we compared INL cell number and IPL thickness, using the number of ONL nuclei for normalization, we found that the IPL had a greater defect than the INL (Fig. 3.1F, G). Taken together, these results demonstrate the significantly decreased
number of amacrine cells in the Foxn4^{-/-} mice, and also point to a decrease in amacrine cell neurites overlap in the IPL.

**Residual amacrine cells in Foxn4^{-/-} mice display normal subtype markers**

In order to better characterize the Foxn4^{-/-} mice, we asked which amacrine cell populations were decreased in these animals. We immunostained retinal cross-sections of knock-out postnatal day 9 mice and from their wildtype littermates, and we found that there was a decreased number of calretinin-immunoreactive amacrine cells (Fig. 3.2A, E), although the difference did not reach statistical significance. There was also no difference in the number of immunoreactive amacrine cells when we immunostained with antibodies against the calcium binding protein parvalbumin (Fig. 3.2B, F), tyrosine hydroxylase (Fig. 3.2C, G) or the gabaergic amacrine cell marker GAD 65/67 (Fig. 3.2D). Thus, the Foxn4^{-/-} mice demonstrate a similar reduction in all subtypes of amacrine cells tested.

**Retinal waves are preserved despite reduction in amacrine cells**

We next asked whether the reduced amacrine cell number in the Foxn4^{-/-} mouse is associated with changes in early retinal activity, specifically in the retinal waves of spontaneous synchronized action potentials observed in the vertebrate retina during development (Meister et al., 1991; Wong et al., 1993). These retinal waves are only detectable before eye opening (Meister et al., 1991; Wong et al., 1993; Feller et al., 1996), and are necessary for the establishment of
a precise retinotopic map (McLaughlin et al., 2003b). Retinal waves are initiated from starburst amacrine cells (Zhou, 1998), a type of displaced amacrine cell in the GCL, and spread among neighboring RGCs and amacrine cells. To examine whether retinal waves are functional in Foxn4−/− retinas, we analyzed the characteristics of retinal waves during development using calcium imaging.

As shown in Fig. 3.3A, we were able to record retinal waves in both Foxn4−/− and wildtype retinas. We found no differences in our ability to record retinal waves from P1 or P2 Foxn4−/− mice and their wildtype littermates. However, by P4-P5, we were not able to detect calcium waves in Foxn4−/− retinas and only rarely in wildtype retinas, likely due to the development of the mesh-like vasculature on the retinal surface that obscures loading and visualization of the Fura-2 dye in the GCL (data not shown).

To examine the properties of the retinal waves in greater detail, we selected all the Fura-2-loaded cells in focus within a field of view. We compared the percentage of cells participating in at least one retinal wave in Foxn4−/− versus wildtype retinas, and we found that the percentage of cells participating in retinal waves was similar in P1 and P2 retinas (Fig. 3.3B). We next analyzed the frequency of the retinal waves by measuring the interval between the spikes. We found no significant differences between P1 or P2 wildtype and Foxn4−/− retinas (Fig. 3.3C). Finally, we examined the waves’ amplitude, including the averaged amplitude of all the waves in one recording (Fig. 3.3D), the maximum amplitude in one field of recording (Fig. 3.3E), and the averaged maximum amplitude of individual cells (Fig. 3.3F), and found no significant differences in any of these
parameters. Taken together, these data demonstrate that, at least through early postnatal ages, there is no significant difference in retinal waves between Foxn4−/− and wildtype mice.

RGCs’ intrinsic axon growth ability in vitro

We next began to investigate whether, in the context of a reduced complement of amacrine cells, RGC axon growth ability or patterning are affected. Previous experiments published by Li et al. (2004) demonstrate that Foxn4 is not expressed in RGCs; however, it is not known whether Foxn4 is expressed in the major target area of the rodent visual pathway, the superior colliculus. We analyzed brain cross-sections of postnatal day 3 wildtype mice and found that Foxn4 could not be detected in the superior colliculus (Fig. 3.4A), whereas the ventricle was immunopositive for the same antibody (Fig. 3.4B). Thus, these data suggest that Foxn4 is not expressed in the superior colliculus, and changes in RGC axon growth or targeting are not likely to result from target-derived changes in the Foxn4−/− mouse.

We have previously demonstrated that amacrine cells are sufficient to signal embryonic RGCs to decrease their intrinsic axon growth ability in vitro; however, it is not known whether amacrine cells are necessary in vivo for embryonic RGCs to undergo this developmental decrease in axon growth ability (Goldberg et al., 2002a). To address this question, we purified labeled RGCs from age P2-P4 Foxn4−/− mice and heterozygous and wildtype littermates, and cultured them on PDL and laminin in a serum-free defined growth media which
strongly promotes RGC survival and axon growth (Fig. 3.5A) (Meyer-Franke et al., 1995; Goldberg et al., 2002b; Goldberg et al., 2002a). After a period of 2 days in culture at clonal density, we fixed the cells and immunostained for the neurite marker Tau to measure different parameters, such as total neurite length, length of the longest neurite and number of branch points. In two independent experiments with at least 70 cells per genotype each, we found that while the rate of axon growth is consistent with previous studies (Goldberg et al., 2002a), there was no significant difference between RGCs from Foxn4⁻/⁻ mice and their wildtype or heterozygous littermates (Fig. 3.5B). Whether the remaining amacrine cells in the Foxn4⁻/⁻ retinas are sufficient to signal RGCs to decrease their intrinsic axon growth ability by P4 as in the wildtype or heterozygous retinas could not be determined from these mice.

**RGCs are delayed in reaching the superior colliculus in Foxn4⁻/⁻ mice**

We next asked whether, in the context of a reduced number of amacrine cells, RGC axons would have an aberrant trajectory along the optic pathway. To address this question, we examined the structures along the optic pathway in Foxn4⁻/⁻ mice by anterograde and retrograde tracing.

First, we injected Alexa-488 and Alexa-594-conjugated cholera toxin B (CTB) intravitreally in Foxn4⁻/⁻ mice and their wildtype littermates of four different ages (P0, P2-P3, P4 and P6). After 24 hours, we collected their eyeballs with the attached optic nerves, obtained transverse cross-sections and examined the intensity of the fluorescent labels within the area of the optic nerve per section.
(Fig. 3.6A). We found no significant differences in the mean fluorescence intensities between the Foxn4−/− and the wildtype pairs between the ages of P0 and P6 (Fig. 3.6B). We also analyzed the morphology of optic nerves by transmission electron microscopy and found that in the Foxn4−/− mice, the tissue architecture was disrupted compared to their wildtype littermates (Fig. 3.6C-D). When we examined axon crossing at the optic chiasm, however, it appeared grossly normal, with most axons crossing and a small percentage remaining ipsilateral (Fig. 3.6E). Thus even when presynaptic amacrine cells are reduced in number, there are likely similar numbers of RGC axons in the optic nerves and, despite mild disruptions in optic nerve structure, axon patterning at the optic chiasm appeared normal.

Next, we retrogradely labeled mice at P1 and P6 with Alexa-594 and -488-CTB, respectively (Fig. 3.7A). First, we observed that the complexity of RGCs’ dendrite morphology in the IPL was reduced in Foxn4−/− compared to wild-type retinas (Fig. 3.7A). We confirmed this impression by immunostaining RGC dendrites with Map2, and again found that RGC dendrite extension was reduced in the context of a reduced number of amacrine cells (Fig. 3.7B).

We quantified the number of RGCs whose axons have reached the superior colliculus and can thus take up the retrograde dyes, and found that at P1, fewer RGCs are retrogradely labeled, but at P6 a similar number of RGCs are retrogradely labeled (Fig. 3.7C). Although there are no differences in the number of RGCs between the Foxn4−/− mice and their wildtype littermates (Li et al., 2004; Fujitani et al., 2006), the number of retrogradely labeled RGCs trended
lower in the Foxn4<sup>-/-</sup> retinas (Fig. 3.7C), raising the hypothesis that fewer RGCs were reaching the superior colliculus in the Foxn4<sup>-/-</sup> mice.

To address this possibility, we examined the fluorescent intensities in cross-sections of the superior colliculus after anterograde tracing of RGC axons (Fig. 3.7D, E). We found a dramatic decrease in labeling from RGC axons in the superior colliculus at early postnatal ages (Fig. 3.7E). Particularly striking was the decrease in RGC axon penetration into the deeper, retinorecipient layers of the superior colliculus (Fig. 3.7E). Together with the retrograde labeling data demonstrating that fewer RGCs reached the superior colliculus, these data demonstrate that in the context of a reduced number of amacrine cells, RGC axons fail to grow properly and penetrate into their major target in early postnatal development, thus supporting a role for presynaptic amacrine cells in RGCs axon growth in vivo.

**DISCUSSION**

These data demonstrate that RGC axon targeting and penetration into the retinorecipient layers is diminished in the setting of a reduced complement of amacrine cells in the Foxn4<sup>-/-</sup> mouse. Foxn4 is a member of the forkhead/winged helix family of transcription factors (Gouge et al., 2001). This evolutionary conserved family of genes have been implicated in a variety of developmental processes (Kaufmann and Knochel, 1996; Carlsson and Mahlapuu, 2002), including cardiac chamber formation in zebrafish (Chi et al., 2008) and neuronal
cell fate determination in *Xenopus* by controlling cell cycle regulation (Hardcastle and Papalopulu, 2000; Burgering and Kops, 2002). In the mammalian retina, Foxn4 is highly expressed in a subset of retinal precursor cells during early development (Gouge et al., 2001) and disruption of its expression leads to a decreased number of horizontal and amacrine cells (Li et al., 2004; Fujitani et al., 2006). There is no Foxn4 expression in RGCs (Li et al., 2004; Fujitani et al., 2006) or in RGC target areas in the superior colliculus, suggesting that the deficits seen in RGC axon outgrowth *in vivo* are attributable to their missing presynaptic partners, retinal amacrine cells.

**Retinal waves are present in early postnatal Foxn4−/− mice**

Our data demonstrated that the retinal waves, recorded with calcium imaging, are still present in the Foxn4−/− retinas at P2. Their spatiotemporal properties, including frequency and amplitude, are almost identical to their littermates, suggesting that these important developmental phenomena do not depend on the full complement of retinal amacrine cells, although it is possible that there is no difference in the initiating starburst amacrine cells in these animals. These experiments did not distinguish between RGC and amacrine cell contribution to the retinal waves, as retrograde labeling of RGCs would be inconsistent given the RGC axon projection to the superior colliculus discovered here. The lack of change in retinal waves does not preclude changes in RGCs’ axon growth; indeed the developmental loss of RGCs’ intrinsic axon growth ability was found to be independent of retinal activity (Goldberg et al., 2002a).
Nevertheless these data demonstrate a component of axon targeting of RGCs that is independent of retinal activity, and may provide clues to differentiating aspects of retinal patterning that are activity-dependent and activity-independent (Katz and Shatz, 1996).

**RGCs’ intrinsic axon growth ability is not modified in the near absence of amacrine cells.**

One of the major questions in the neuroscience field is why neurons of the CNS fail to regenerate their axons after injury. We previously found that amacrine cells can signal embryonic RGCs to turn off their intrinsic growth ability during development (Goldberg et al., 2002a) and thus we had initially hypothesized that in the context of a reduced number of amacrine cells, RGCs might not decrease their axon growth ability on time. We purified RGCs from postnatal Foxn4−/− mice and found no difference in their ability to grow axons, compared to RGCs from wildtype littermates. It is possible that that signal from amacrine cells that tells RGCs to turn off their intrinsic axon growth ability could have been already delivered by the residual subset of amacrine cells by P2-P4. By P0, however, over 95% of RGC axons have reached their targets in the superior colliculus (Hofbauer and Drager, 1985).

**Developmental delay in the normal projection of RGCs to the superior colliculus.**

Seeing no difference in the RGCs' intrinsic axon growth ability in Foxn4−/− mice, we asked whether the RGCs would project their axons normally along the
optic pathway. To address this question, we took advantage of the RGCs' ability to transport dyes antero and retrogradely, and conducted experiments to study the number of RGCs labeled by injecting CTB in the superior colliculus and vice versa, the fluorescence intensity in the superior colliculus after injecting a dye in the retina. Both a decreased number of labeled RGCs (while the total number of RGCs was similar, Fig. 3.1C) and less fluorescence intensity in superior colliculi of the Foxn4^-/- mice compared to their wildtype or heterozygous littermates, confirm that in the context of a decreased number of amacrine cells, RGCs do not project their axons to the right place at the right time. Another possibility is that RGC axons have a defect that prevents the proper transport of the dye. Here we show by electron microscopy that the morphology of the optic nerve is altered in the Foxn4^-/- retina.

Taken together, these data suggest that Foxn4 is required for proper wiring of the retino-collicular projection during development, and suggest a novel role for the presynaptic partner in controlling the postsynaptic neurons' developmental wiring in vivo. Future experiments directed at understanding the molecular mechanism may further enhance our understanding of this potentially novel mechanism of controlling patterning in the developing nervous system.
CHAPTER FIGURES

A

# nuclei in GCL/100 µm

Central  Peripheral

WT  KO

** * *

B

# nuclei in INL/100 µm

Central  Peripheral

WT  KO

#

C

IPL thickness, µm

Central  Peripheral

WT  KO

*** *

D

# nuclei in ONL/100 µm

Central  Peripheral

WT  KO


E

DAPI  Syntaxin  Vc1.1  Merged

foxp4 KO  wild type

F

IPL thickness/ # of nuclei / 100 µm in the ONL

Central  Peripheral

WT  KO

*** **

G

# of nuclei in INL/ONL

Central  Peripheral

WT  KO

*
**Figure 3.1** Decreased number of amacrine cells in the Foxn4−/− retinas. (A) Quantitative analysis of the number of nuclei in the ganglion cell layer (GCL) and in the inner nuclear layer (INL) (B) of Foxn4−/− mice and their wild-type littermates showed a significant decrease in the number of DAPI-labeled nuclei along the lines traced in these layers both in central and peripheral retina. (C) The thickness of the inner plexiform layer (IPL) was decreased in Foxn4−/− retinas, while the number of nuclei in the outer nuclear layer (ONL) was similar (D). (E) Retinal cross-sections from postnatal day 6 mice were immunostained with two amacrine cell markers, syntaxin and VC1.1. Amacrine cells are decreased by 50-75% in the Foxn4−/− mouse. (F) Quantification of the IPL thickness in relationship to the number of nuclei in the ONL. (G) Quantification of the number of nuclei in the INL in relationship to the number of nuclei in the ONL; #: p<0.06; *: p<0.05; **:p<0.01; ***: p<0.005, Student’s t-test; N=4 retinas (P8-P9), 3 measurements per retina. Error bars: SEM.
Figure 3.2 Characterization of amacrine cells in the Foxn4^-/- retinas. Postnatal day 9 retinas were immunostained with the antibodies as labeled (A-D). Quantification of the percentage of immunopositive amacrine cells of the Vc1.1 (+) population. No significant differences were detected between Foxn4^-/- mice and their wild-type littermates (E-G); N=2 retinas, error bars: SEM. Calretinin: p<0.1; Student’s t-test.
Figure 3.3 Retinal waves. (A) Probability of recording waves in both WT and KO retinas across different age groups. Each circle represents one retina. At P4 and P5, no waves could be recorded in the Foxn4 KO retinas. (B-F) There was no statistically significant difference among WT and KO for the different parameters measured: fraction of cells participating in at least one calcium wave (B), retinal waves’ interval (C) and amplitude (E), including the average amplitude of all waves in one recording (F) and the maximum amplitude of individual cells (D). (Student’s t-test; the data shown are Mean ± SD.)
Figure 3.4 Foxn4 immunostaining in the superior colliculus. (A) Foxn4 expression is not detectable in the superior colliculus of P3 wildtype mice, while there is immunoreactivity in the ventricle (B).
Figure 3.5 Analysis of RGC axon growth in vitro. (A) The day before the experiment and after genotyping, RGCs were labeled by intravitreous injection of CTB, using different fluorophores for KO and WT or HET mice. (B) The following day, dye-labeled animals (P2 and P4) were sacrificed and RGCs were purified, cultured in serum-free media and immunostained at 2DIV with anti-Tau. Automated tracing was performed on 900+ wild-type RGCs and at least 70 RGCs from Foxn4<sup>−/−</sup> mice at each age. Two independent experiments were performed. Error bars: SEM.
Figure 3.6 Optic nerve morphology and number of RGC projections. (A) Postnatal mice of different ages ranging from P0 to P6 were injected CTB in the vitreous. 6 hours post-injection, animals were euthanized and optic nerves were collected and cryosectioned. Shown are example cross-sections of P4 optic nerves. The fluorescence intensity was quantified taking the area of the nerve into consideration (B). (C) Electron micrograph of optic nerve shows defects in the axon morphology in Foxn4⁻/⁻ mice compared to their wildtype littermates (D). (E) Axon crossing at the optic chiasm, appeared grossly normal in Foxn4⁻/⁻ mice, with most axons crossing contralaterally and a small percentage remaining ipsilateral (arrow).
Figure 3.7 RGC axons in vivo. (A) RGCs from P1 and P6 mice were retrogradely labeled and the animals were euthanized 24 hours later. The qualitative analysis of the retinal cross-sections shows a decrease in the number of labeled RGCs in the Foxn4<sup>−/−</sup> retinas. Quantitative analysis of the P7 retina confirms the findings (C). (B) Postnatal day 7 Foxn4<sup>−/−</sup> and heterozygous mice were injected 1% CTB in the superior colliculus. Animals were sacrificed the next day and the retinas were collected to perform immunostaining with Map2 antibody. (D) The brains of the same animals in (A) were collected, sectioned and mounted on glass slides with DAPI. (E) Quantification of the mean fluorescence intensity reveals a decrease in the number of labeled projections reaching the superior colliculus by P6 in the Foxn4<sup>−/−</sup> mice.
REFERENCES


Famiglietti EV, Jr., Kolb H (1975) A bistratified amacrine cell and synaptic
circuitry in the inner plexiform layer of the retina. Brain Res 84:293-300.

profiling of purified rat retinal ganglion cells. Invest Ophthalmol Vis Sci
45:2503-2513.

for cholinergic synaptic transmission in the propagation of spontaneous

Filbin MT (2006) Recapitulate development to promote axonal regeneration:
good or bad approach? Philos Trans R Soc Lond B Biol Sci 361:1565-
1574.

traumatic ganglion cell death and promotes axonal regeneration both in

ganglion cells to a robust growth state in vivo: gene expression and

Frisen J, Yates PA, McLaughlin T, Friedman GC, O’Leary DD, Barbacid M (1998)
Ephrin-A5 (AL-1/RAGS) is essential for proper retinal axon guidance and
topographic mapping in the mammalian visual system. Neuron 20:235-
243.

Fujitani Y, Fujitani S, Luo H, Qiu F, Burlison J, Long Q, Kawaguchi Y, Edlund H,
MacDonald RJ, Furukawa T, Fujikado T, Magnuson MA, Xiang M, Wright
CV (2006) Ptf1a determines horizontal and amacrine cell fates during
mouse retinal development. Development 133:4439-4450.

Fung SC, Kong YC, Lam DM (1982) Prenatal development of gabaergic,
glycinergic, and dopaminergic neurons in the rabbit retina. J Neurosci
2:1623-1632.

reach a threshold required to overcome inhibition by MAG through
extracellular signal-regulated kinase-dependent inhibition of

Gauthier LR, Charrin BC, Borrell-Pages M, Dompierre JP, Rangone H,
Cordelieres FP, De Mey J, MacDonald ME, Lessmann V, Humbert S,
Saudou F (2004) Huntingtin controls neurotrophic support and survival of
neurons by enhancing BDNF vesicular transport along microtubules. Cell


APPENDIX: Disease Gene Candidates Revealed by Expression Profiling of Retinal Ganglion Cell Development

PAPER ABSTRACT

To what extent do postmitotic neurons regulate gene expression during development or after injury? We took advantage of our ability to highly purify retinal ganglion cells (RGCs) to profile their pattern of gene expression at 13 ages from embryonic day 17 through postnatal day 21. We found that a large proportion of RGC genes are regulated dramatically throughout their postmitotic development, although the genes regulated through development in vivo generally are not regulated similarly by RGCs allowed to age in vitro. Interestingly, we found that genes regulated by developing RGCs are not generally correlated with genes regulated in RGCs stimulated to regenerate their axons. We unexpectedly found three genes associated with glaucoma, optineurin, cochlin, and CYP1B1 (cytochrome P450, family 1, subfamily B, polypeptide 1), previously thought to be primarily expressed in the trabecular meshwork, which are highly expressed by RGCs and regulated through their development. We also identified several other RGC genes that are encoded by loci linked to glaucoma. The expression of glaucoma-linked genes by RGCs suggests that, at least in some cases, RGCs may be directly involved in glaucoma pathogenesis rather than indirectly involved in response to increased intraocular pressure. Consistent with this hypothesis, we found that CYP1B1 overexpression potentiates RGC survival.
PAPER INTRODUCTION

The molecular basis underlying the complex postmitotic development of a neuron is essentially unknown. For example, most rat retinal ganglion cells (RGCs) are born between embryonic day 13 (E13) and E18, extend their axons toward their targets between E14 and postnatal day 2 (P2), elaborate dendrites into the inner plexiform layer of the retina from E16, begin to receive synapses late embryonically, undergo a period of naturally occurring cell death between P2 and P5, and allow optic nerve oligodendrocytes to begin myelination after P7.

How much of the molecular control of the timing of these dynamic processes is attributable to developmental changes in gene expression of the neuron, either as the result of an intrinsic program or in response to signaling from its neighbors? In some cases, genes important for normal RGC development are expressed constitutively. For instance, as soon as they are generated by their precursor cells, RGCs express Eph receptors that are used 1–2 weeks later for topographic map formation in their targets in the superior colliculus and then continue to express these genes into adulthood (McLaughlin et al., 2003a; Rodger et al., 2005). Other changes in RGC phenotypes may depend on developmental regulation of RGC gene expression. For example, RGCs undergo a developmental loss of their intrinsic capacity for rapid axon growth just after their axons reach their targets in the superior colliculus. Amacrine cells can signal embryonic RGCs to turn off their intrinsic capacity for axon growth, and this amacrine signal is dependent on new gene expression by RGCs (Goldberg et al., 2002a).
To build new hypotheses for how dynamic RGC processes such as axon and dendrite growth, developmental cell death, synapse formation, and other neuron–glial interactions are timed and regulated, here we have characterized the RGC transcriptome throughout development. Gene expression profiling using microarrays and serial analysis of gene expression (SAGE) has been used previously to characterize gene changes occurring during development in various tissues or after injury, but the multiplicity of cell types in most tissues has in many cases limited the utility or interpretation of the data. For example, SAGE analysis of the mouse retina did not reveal any genes limited through development to RGCs, most likely because RGCs make up only 0.5% of the neurons in the retina (Blackshaw et al., 2004).

Here we take advantage of our ability to purify RGCs to homogeneity at different ages to better define the transcriptional changes that may account for the postmitotic differentiation program through RGC development. We found that the RGC transcriptome is surprisingly dynamic from E17 to P21. We identified changes in gene expression of as much as 100-fold that occur concurrently with major developmental events in the life of an RGC. We found that the gene changes accompanying normal RGC development are not mimicked by the gene changes that occur after RGC axotomy and attempted axon regeneration. Finally, we identified several genes linked to glaucoma that were unexpectedly highly expressed by RGCs and demonstrate that CYP1B1 (cytochrome P450, family 1, subfamily B, polypeptide 1) plays a role in RGC survival in vitro. The gene database we generated provides an invaluable new resource for generating
testable hypotheses about how RGCs develop, why they fail to regenerate, and why RGCs die in glaucoma and other diseases.

MATERIALS AND METHODS

Purification of retinal ganglion cells

RGCs from E17 through P21 Sprague Dawley rats were purified by sequential immunopanning to 99.5% purity and cultured on poly-D-lysine (PDL) (70 kDa, 10 µg/ml; Sigma, St. Louis, MO) and laminin (2 mg/ml; Telios/Invitrogen, Carlsbad, CA) in serum-free defined medium as described containing BDNF (50 ng/ml), CNTF (10 ng/ml), insulin (5 µg/ml), and forskolin (5 µM) (Barres et al., 1988; Meyer-Franke et al., 1995). Detailed protocols are available on request.

RNA preparation, microarray hybridization, and data analysis

RGCs from 13 different ages of Sprague Dawley rats were purified and allowed to recover for 1 h at 37°C, after which total RNA was extracted using the RNeasy kit (Qiagen, Hilden, Germany). RNA quality was assessed by spectrophotometry and gel electrophoresis. Total RNA (1 µg) was reverse transcribed into cDNA, amplified once by in vitro transcription, reverse transcribed into cDNA, and then used to generate biotin-labeled cRNA, according to the protocols of Affymetrix (Santa Clara, CA). Fragmented cRNA was hybridized onto rat genome U34A, U34B, and U34C arrays (Affymetrix) containing 26,000 probes against ~13,000 genes. Scanned output files were analyzed with Microarray Suite 5.0 (Affymetrix) as well as by ANOVA and
normalized to an average intensity of 1500. Subsequent analysis using Microarray Suite 5.0, Excel (Microsoft, Seattle, WA), and SAS 8.0 (SAS Institute, Cary, NC) for ANOVA confirmation of statistical reproducibility is described in Results. Time course data for all probes are available on-line at

http://www.jneurosci.org/content/vol27/issue32/images/data/8593/DC1/Supplemental_Table_1_-_RGC_Gene_Expression_Data.txt.

**Quantitative real-time reverse transcription-PCR**

Total RNA was extracted from purified E20 and P8 RGCs as described above, of which 1 µg was reversed transcribed into cDNA (iScript; Bio-Rad, Hercules, CA). Equal amounts of cDNA were further amplified by real time-PCR (iQ SYBR Green supermix; Bio-Rad). We used 18S ribosomal RNA as an internal reference. Primers used were as follows: matrix Gla protein (Mgp) forward, GCCCTGATCTACGGGTACAA; Mgp reverse, GAACAAGCAACGAACGAA; connective tissue growth factor (Ctgf) forward, CAGGGAGTAAGGGACACGAA; Ctgf reverse, TCCCACAGCAGTTAGGAACC; Gp46 forward, GGTACTGCGGAGAAGCTGAG; Gp46 reverse, CCAAGGGTGACAGGAGGATA; 18S forward, GAACTGAGGCCATGATTAAGAG; and 18S reverse, CATTCTGGCAAATGCTTTC. Reactions were run with five replicates per primer pair, and repeated at least three times on different days.
Western blotting and immunofluorescence

Western blotting was performed using standard protocols. Approximately 15 µg of acutely isolated E20 and P8 RGC cell lysates collected in radioimmunoprecipitation assay buffer were transferred to 0.45 µm nitrocellulose (Bio-Rad) and incubated overnight at 4°C in rabbit anti-CYP1B1 antiserum (3 µg/ml; Alpha Diagnostics, San Antonio, TX), rabbit anti-optineurin antiserum (5 µg/ml; Caymen Chemical, Ann Harbor, MI), or chicken anti-cochlin antiserum (1:500) (Robertson et al., 2001), followed by 2 h with anti-rabbit or anti-chicken horseradish peroxidase secondary antibody (1:10,000; Millipore, Billerica, MA). All incubation was performed in 5% milk. Detection was performed using ECL (GE Healthcare, Little Chalfont, UK).

For immunostaining of retina, retinal tissues were dissected from freshly killed E20, P8, and adult (P21) Sprague Dawley rats and immediately fixed in 4% paraformaldehyde for 1 h. The tissues were cryoprotected in 30% sucrose, snap frozen in mounting medium (OCT, Tissue-Tek; Miles, Elkhart, IN), and 8–12 µm sections were cut and mounted on aminoalkylsilane-coated slides (Sigma). Sections were postfixed in 4% paraformaldehyde for 10 min and then blocked and permeabilized with 20% goat serum and 0.4% Triton X-100 for 30 min. Retinal tissues were incubated with anti-lysyl oxidase (1:100; generous gift from K. Csiszar, University of Hawaii, Honolulu, HI) and the same anti-CYP1B1, anti-optineurin, or anti-cochlin antibody as described above at 5 µg/ml, 10 µg/ml, and 1:1000 dilutions, respectively. Secondary detection was performed with fluorescence antibodies (1:500 dilution; Millipore) using anti-rabbit (for CYP1B1,
optineurin, and lysyl oxidase) or anti-chicken antibody (for cochlin). The slides were mounted in Vectashield with 4',6'-diamidino-2-phenylindole, sealed with nail polish, and examined in a Nikon (Tokyo, Japan) Diaphot fluorescence microscope.

For immunoreactivity of RGCs, E20 and P8 RGCs were purified and cultured on laminin-coated glass coverslips in serum-free growth media for 24 h. Cultures were maintained at 37°C in a humidified environment of 10% CO₂/90% O₂. Cell fixation, membrane permeabilization, and antibody incubation were performed as above.

**Analysis of human glaucoma genes**

We identified glaucoma loci at the National Center for Biotechnology and Information Online Mendelian Inheritance in Man Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=omim). For each glaucoma locus, we generated a list of candidate genes encoded between the human genomic linkage analysis markers described in the appropriate reference. Using Netaffx Analysis Center (Affymetrix), we determined whether these genes were expressed by at least 10% of RGC samples in the microarray data and then further identified the subset of genes that change more than threefold during development (E17 to P21).
RGC transfection and survival assay

Full-length cDNA constructs for mouse CYP1B1 (clone ID 30007248), cochlin (clone ID 6306601), and rat optineurin (clone ID 7111026) (all from Openbiosystems, Huntsville, AL) were verified by sequencing and amplified by Maxi-preps (Qiagen). Small interference RNA (siRNA) for rat CYP1B1 (catalog number L-087731-00; Dharmaco, Lafayette, CO) concentration and quality were assessed by spectrophotometry. Transfection of cDNA or siRNA constructs into RGCs was achieved using Nucleofector for primary neurons ("G13" setting; Amaxa, Gaithersburg, MD). Briefly, ~3.5 x 10^5 purified RGCs were electroporated, allowed to recover at 37°C for 1 h, and subsequently cultured on PDL- and laminin-coated six-well plates for 72 h. Transfection efficiency was separately measured with a supplied control pmaxGFP or mCherry plasmid.

Survival was determined by treatment of RGCs with a mammalian live/dead cell assay kit containing calcein AM and ethidium homodimer (Invitrogen), fluorescent indicators of metabolically active and membrane-permeant cells, respectively, for 30 min, after which the cells were counted in 10 random, independent fields by an investigator masked to each condition on an inverted fluorescent microscope at 20x. A two-proportion Z test between experimental and control conditions was used to test for significance.
RESULTS

Retinal ganglion cell gene expression

RGCs were purified to homogeneity by immunopanning from 13 ages: E17, E18, E19, E20, E21, P0, P1, P2, P5, P8, P11, P16, and P21. Three to four biological replicates were prepared from each age and processed independently for microarray analysis using Affymetrix U34A–U34C rat genome arrays. Intersample variability was very low, with a Pearson's correlation coefficient of $r^2 \geq 0.90$ for same-age samples (Fig. 4.1A); comparing E17 to P21 samples, in contrast, yielded $r^2$ values of $\sim$0.1–0.2.

Using the algorithm in Microarray Suite 5.0 to determine whether probed genes were "absent" or "present," we found that 33% of probed genes were expressed in RGCs throughout the entire time course, an additional 29% were expressed in a subset of time course samples, and 38% were not detected in RGCs at all (Fig. 4.1B). We found in these data that probes began to be called present at signal strengths $\sim$1000 (arbitrary units) (supplemental Fig. 4.1), although signal strength was not a criterion we used to filter the data further. We analyzed only those 14,166 probes that the Affymetrix Microarray Suite 5.0 algorithm called present in at least 10% of the RGC samples from here forward, making sure that probes were also reproducibly present in RGC samples of at least one age. We found a remarkable number changed in their levels of expression through development: 3621 probes (26%) changed at least threefold, 474 (3.3%) changed at least 10-fold, and 175 (1.3%) changed at least 20-fold (Fig. 4.1C and supplemental Table 4.2, available at
We compared the top changers in RGCs with the top changers published for striatal (Lobo et al., 2006) and cortical (Arlotta et al., 2005) projection neurons and found that each population developmentally regulates very different genes. Of the 35 reported genes developmentally regulated between P20 and adult striatal neurons, only three were found in our data to be developmentally regulated in RGCs between E20 and P21 (heat shock 70 kDa protein 1A, ryanodine receptor 2, and calbindin 1). Similarly, of the 27 reported genes between E18 and P14 in corticospinal motor neurons that were found in the RGC GeneChip dataset, only two were developmentally regulated in RGCs (Purkinje cell protein 4 and neurofilament-H).

We also compared P21 RGC gene expression with adult retinal gene expression published previously (Vazquez-Chona et al., 2004) using triplicate biological samples and data from the Affymetrix U34A GeneChips (~8800 probes). Of the 3566 genes present in at least two of three P21 RGC samples, most were also observed in retina (Fig. 4.1D). We identified 28 genes at least 20-fold more highly expressed in RGCs compared with total retina by microarray analysis (Table 4.1) and 1016 genes at least threefold greater in RGCs compared with total retina, although many of these may not be good candidates for RGC-specific genes.

To classify RGC gene expression further, we analyzed the 8557 probes annotated by gene ontology and fit 6612 of them into 27 partially overlapping categories. We found that RGCs expressed between 39 and 80% of the probes in these gene ontology categories (Table 4.2). There was not a statistically significant difference in the likelihood of expressing a greater or lesser
percentage of genes in any one category. We then examined what gene ontologies were most likely to contain genes that change at least threefold across RGC development (Table 4.2) and found statistically significant differences in the fractions of genes changing in different categories. Specifically, several ontologies were less likely to contain genes that changed through RGC development, including mitochondrion/mitochondrial (15%), nicotinamide adenine dinucleotide (nadh)/nadph (8%), proteasome (0%), RNA (19%), and ubiquitin (13%), and two stood out as more likely to contain developmentally regulated genes, neurotransmitter (50%) and synapse/synaptic (42%). Thus, protein functions relating to energy metabolism and protein degradation pathways remained fairly constant through the postmitotic maturation of RGCs, whereas synaptic communication played the most dramatic role in developmental changes in RGC gene expression.

We were interested in a cluster of genes related to extracellular matrix and chose to validate the gene expression data from the microarrays by quantitative real-time reverse transcription (RT)-PCR and immunostaining. We confirmed that Mgp, Ctgf, and the collagen-binding protein Gp46 change in the same direction in the GeneChip as by quantitative real-time RT-PCR (Fig. 4.2A), and we found a developmentally regulated increase in lysyl oxidase protein expression in vivo and in vitro by immunostaining (Fig. 4.2B). As others have found, the scale of fold change by microarray and by real-time RT-PCR was often different, although the direction of fold change was always the same. These data, together with the
immunostaining and Western blotting for glaucoma-related genes presented below (see Fig. 4.6), help validate the microarray data.

**Clustering by samples and by genes**

We next asked at what age(s) do RGCs undergo their greatest changes in gene expression? We applied non-negative matrix factorization (NMF) to cluster RGC samples into age ranges (Brunet et al., 2004). First, the samples clustered remarkably well by age, suggesting that biological variability was greater between than within age group samples. A two-cluster solution split 42 RGC samples exactly at the border between P2 and P5, a three-cluster solution split RGC development at E21 and P5, and four- and five-cluster solutions added additional splits between E19 and P16 with a maximum of 5 of 42 samples realigned for consistency (Fig. 4.3). Although we had an interest in the developmental loss of axon growth ability that occurs at P0 (Goldberg et al., 2002a), it is apparent from these data that many of the gene changes during development may be associated with other biological processes, such as synaptogenesis and neurotransmission. Thus, RGCs appear to undergo their most dramatic change in overall gene expression at P2, and these data furthermore suggest that the developmental age of an RGC may be reliably determined by its gene expression profile.

We used hierarchical clustering to ask whether there were repeated patterns of RGC gene expression regulation throughout the time course. Probes expressed in a minimum of 10% of samples and exhibiting a fold change more
than three were analyzed using the Affymetrix Database Mining Tool. A four-cluster solution yielded a group of genes that decreased in expression through development, two groups that increased through development, and an interesting group that spiked in the period around birth (Fig. 4.4) (supplemental Table 4.3, available at http://www.jneurosci.org/cgi/data/27/32/8593/DC1/2). Clustering solutions allowing more than four clusters yielded subclusters primarily derivative of the two patterns shown in Figure 4.4 (A, B).

**Intrinsic aging versus extrinsic regulation of RGC gene expression**

We next asked whether RGCs regulate their gene expression similarly *in vivo* and *ex vivo* in purified cultures. We purified P7–P8 RGCs and aged them in growth media for 8–10 d *in vitro*, to the age they would have been P16 *in vivo*. We then compared these aged RGCs with acutely purified P8 and P16 RGC using GeneChips, analyzing triplicate or quadruplicate biological replicates for each of these three groups. Of 26,256 probes, the MicroArray Suite (Affymetrix) algorithm detected 14,942 (57%) as present in at least one biological sample. We found that 941 of 14,942 probes (6.3%) changed at least threefold between acutely purified P8 and P16 RGCs (aged "*in vivo*") or between acutely purified P8 RGCs and P8 RGCs aged *in vitro*. Of these 941 probes, only 96 (10.2%) changed coordinately in RGCs aged *in vitro* versus *in vivo*, whereas 168 probes changed only *in vivo*, 666 changed only *in vitro*, and 11 changed in opposite directions in the two conditions. Thus, at least by analysis of gene expression profiles, RGCs aged *in vitro* do not closely mimic RGCs aged *in vivo*, although it
is possible that placing the cells in culture interfered with a cell-autonomous progression in gene expression that RGCs were expressing \textit{in vivo}. This suggests that many of the postmitotic changes in RGC expression are dependent on signals from the environment \textit{in vivo} not found in cell culture.

\textbf{Expression of genes regulated both during development and after regeneration}

RGCs normally fail completely to regenerate after axon injury in the optic nerve, but a small subset of RGC axons can be induced to reextend past the injury site in response to lens injury, which recruits macrophages and elicits release of a novel trophic factor for RGCs (Fischer et al., 2000; Leon et al., 2000; Yin et al., 2003; Fischer et al., 2004; Yin et al., 2006). By fluorescence-activated cell sorting retrogradely labeled RGCs, the genes regulated by lens injury after axotomy were compared with those regulated by axotomy alone using microarray analysis (Fischer et al., 2004). We asked whether any of the 73 identified genes regulated at least threefold after axotomy plus lens injury were also regulated during RGC development. Sixty genes were represented in our dataset, of which 46 (77\%) were expressed by developing RGCs, and 34 (74\% of those expressed) demonstrated at least a threefold developmental regulation of expression between E17 and P21 (Fig. 4.5A. We divided these 34 genes into four groups according to whether they were upregulated or downregulated in each dataset (Fig. 4.5B). Because RGCs decrease their axon growth ability during development, we hypothesize that genes that are developmentally downregulated could be stimulatory for axon growth; 10 of 13 of these are
upregulated after axotomy with lens injury (Fig. 4.5). Conversely, we hypothesize that genes that are developmentally upregulated may be inhibiting RGC axon growth; 12 of 21 of these are downregulated after axotomy with lens injury. A caveat to this analysis is that the previous data did not identify genes expressed in the subset of RGCs that successfully regenerated into the optic nerve, but rather the genes expressed in all RGCs under conditions that stimulate a subset to regenerate (Fischer et al., 2004). Thus, 22 (65%) of 34 genes regulated during development and after axotomy plus lens injury are regulated inversely in these two conditions and may be critical candidates when exploring the link between developmental and regenerative axon growth.

**Expression of glaucoma candidate genes by RGCs**

We unexpectedly identified RGC expression of three genes, CYP1B1, optineurin, and cochlin, in which mutations are associated with glaucoma (Stoilov et al., 1997; Rezaie et al., 2002; Bhattacharya et al., 2005). We found that optineurin and cochlin mRNA were upregulated during development (1.86- and 15.2-fold, respectively), and CYP1B1 mRNA was downregulated (27.1-fold) through this time course (Fig. 4.6A).

Immunostaining for these three proteins in the neural retina all showed significant staining in the RGC layer (Fig. 4.6B). Bright, punctuate staining of RGCs was most prominent for CYP1B1 in embryonic retina and for optineurin in postnatal retina. For cochlin and CYP1B1, immunoreactivity was also intense in
the pigmented retinal epithelium, consistent with previous immunolocalization studies in the eye (Bhattacharya et al., 2005; Doshi et al., 2006).

We confirmed RGC expression of these three proteins by Western blot on protein lysates from purified RGCs (Fig. 4.6C). A doublet band was observed at 80 and 65 kDa for CYP1B1, with higher protein expression seen in embryonic RGCs. A doublet band was observed at 40 and 60 kDa for optineurin, with higher protein expression in postnatal RGCs. For cochlin, a single band of ~60 kDa was observed, with slightly higher protein expression in postnatal RGCs. Similarly, in purified RGCs, immunostaining showed predominantly cytoplasmic localization for CYP1B1 in embryonic and postnatal RGCs. Cochlin and optineurin demonstrated a more diffuse localization but with increasing cytoplasmic staining for both proteins in postnatal RGCs (Fig. 4.6D). These data further confirmed the developmental upregulation of cochlin and optineurin and the developmental downregulation of CYP1B1 expression in RGCs, consistent with the respective change of mRNA levels observed in the microarray data.

At least 13 other human genomic loci have been associated with glaucomatous disease by linkage analyses, although the causative genes in these loci are not known. We hypothesized that RGCs may express a subset of genes from these loci and found that, of the 1590 genes expressed in these 13 genomic loci, probes were identifiable in our database for 479 rat homologs. Of these 479 genes, RGCs expressed 343 (72%), and, of these, 109 (32%) were developmentally regulated at least threefold (supplemental Table 4.4, available at
http://www.jneurosci.org/cgi/data/27/32/8593/DC1/3). A subset of these is known to be associated with apoptosis by gene ontology designation (supplemental Table 4.5, available at http://www.jneurosci.org/cgi/data/27/32/8593/DC1/4); many have as yet unknown functions.

**Effect of glaucoma-associated genes on RGC survival**

That RGCs highly express and developmentally regulate several candidate glaucoma genes suggests that some of these genes may function cell autonomously in normal or diseased states. One such function may be to directly influence RGC survival. To test this hypothesis, we overexpressed these three candidate genes in both postnatal and embryonic RGCs using electroporation. There was no consistent effect of overexpression of optineurin or cochlin in these experiments (Fig. 4.7A, B). We found that overexpression of CYP1B1, however, modestly but significantly improved survival in both embryonic and postnatal RGCs (Fig. 4.7A, B). The effect was slightly stronger in postnatal RGCs, which express lower levels of CYP1B1 endogenously *in vivo* and *in vitro* (Fig. 4.6). CYP1B1 encodes a mono-oxygenase of the P450 superfamily and catalyzes the rate-limiting step in retinoic acid (RA) synthesis. It is possible that RA may either augment or be a neuroprotective effector of CYP1B1. To test the relationship between RA and CYP1B1 on RGC survival, we transfected CYP1B1 into cultured RGCs in various RA concentrations. We found that, at 10 µM retinoic acid or above, survival was modestly improved in CYP1B1-transfected RGCs for both E20 and P8 RGCs compared with control RGCs (data not shown).
We next asked whether gene knockdown of CYP1B1 by siRNA would have the opposite effect on embryonic RGC survival. We found that CYP1B1 but not control siRNA decreased embryonic RGC survival (Fig. 4.7C). We further cotransfected the plasmids with a mCherry reporter construct to confirm successful transfection and to determine the percentage of live cells that are overexpressing our plasmids of interest. We found that there is a significantly greater portion of surviving postnatal RGCs that overexpress CYP1B1 than siRNA–CYP1B1 or the reporter construct alone. Thus, our results suggest that CYP1B1 overexpression promotes RGC survival \textit{in vitro} and that it may normally be neuroprotective in RGCs.

**DISCUSSION**

**The postmitotic RGC transcriptome is highly dynamic**

By purifying RGCs, which normally represent fewer than 0.5% of the cells in the retina, we found we could characterize RGC gene expression through development from E17 to P21. These data greatly extend the single time point snapshots of gene expression we and others have published previously for RGCs (Farkas et al., 2004; Fischer et al., 2004; Ivanov et al., 2006) and allowed comparison with development surveys of gene expression in other CNS neurons, including striatal (Lobo et al., 2006) and cortical (Arlotta et al., 2005) projection neurons. Interestingly, it appears that the genes that are developmentally regulated in each of these three populations are almost entirely different. As seen
in these two previous papers, the ability to survey RGC transcripts without the >100-fold dilution of other retinal cells present likely allowed an increased sensitivity and specificity for detecting RGC gene expression and generated candidate genes that are highly enriched in RGCs and may yield RGC-specific markers. A caveat to these data are the comparison of young adult (P21) RGCs to adult retinas, and RGC gene expression appears likely to change between these time points based on the dramatic changes seen through the rest of RGC development. Whether these genes are also present in adult RGCs remains to be elucidated.

Although these neurons are postmitotic, their transcriptome proved highly dynamic through development, with 26% of genes changing expression level at least threefold through this period and >3% changing at least 10-fold. Interestingly, we found that developmental changes in RGC gene expression are primarily dependent on environmental signals, whereas RGCs were able to regulate relatively few of the same genes by aging in vitro in purified cultures. Thus, the postmitotic transcriptome is highly dynamic and appears to be highly dependent on neighboring cells. By coculturing RGCs with other purified populations of CNS neurons and glia from the retina, optic nerve, and targets in the brain, we may discover which cell types are responsible for regulating at least a subset of RGC gene expression changes.
**RGCs express glaucoma candidate genes**

This study identified RGC expression of optineurin, in which mutations have been identified in a subset of familial primary open-angle glaucoma (POAG) patients with linkage to the GLC1E locus (Rezaie et al., 2002). Our findings that optineurin mRNA and protein expression increase through RGC development is consistent with previous reports of optineurin protein expression in adult mouse RGCs (Rezaie and Sarfarazi, 2005; De Marco et al., 2006). Optineurin binds to Huntingtin (Faber et al., 1998), a protein with a recently discovered role in axonal BDNF transport and survival signaling (Gauthier et al., 2004). In addition, in cell lines, optineurin translocates to the nucleus in response to proapoptotic oxidative stress, and overexpression of the wild-type but not a mutated transcript is protective in these assays (De Marco et al., 2006). Thus, loss of normal optineurin function may lead to attenuated axonal trophic transport in RGCs and secondary neuronal degeneration, consistent with the characteristic axon loss seen in glaucoma.

Our data also revealed RGC expression of cochlin, a gene whose protein deposits were found in higher levels in the trabecular meshwork (TM) of POAG patients and glaucomatous DBA/2J mice (Bhattacharya et al., 2005). Mutant cochlin in the TM, absent in nonglaucomatous mice and healthy humans, may obstruct aqueous humor drainage and subsequently elevate increased intraocular pressure (Bhattacharya et al., 2005). The abnormal cochlin aggregates seen in some adult-onset glaucomas and the developmental
upregulation of normal cochlin expression by RGCs seen in our study suggest
that a source of mutant cochlin deposits may come from the RGCs themselves.

We also found RGC expression of CYP1B1, the gene linked to the GLC3A primary congenital glaucoma locus (Stoilov et al., 1997). Previous work has localized CYP1B1 expression to the anterior segment of the eye, and a CYP1B1 knock-out mouse demonstrates anterior segment abnormalities (Libby et al., 2003). CYP1B1 expression was also previously localized to early neural layer of the optic cup (Stoilov et al., 2004), but our data extend these observations to localize the protein to RGCs and furthermore demonstrate that the message and protein are upregulated in RGCs through development.

**CYP1B1 enhances RGC survival**

We furthermore found that overexpressing CYP1B1 enhanced RGC survival, and knocking down CYP1B1 appeared to decrease RGC survival *in vitro*. CYP1B1 encodes a mono-oxygenase of the P450 superfamily and catalyzes the rate-limiting step in RA synthesis, oxidizing retinol to all-trans retinal (Chen et al., 2000). Interestingly, RA participates in cell proliferation and polarity establishment in early eye development (Sen et al., 2005) and may have antiapoptotic activity in the adult CNS by inhibiting JNK (c-Jun N-terminal protein kinase) activation (Ahn et al., 2005) or by inhibiting microglial expression of tumor necrosis factor-α (Dheen et al., 2005). The high embryonic expression of CYP1B1 observed in our study suggests that it may be an important source of RA for RGCs during embryonic and early postnatal development, especially considering
that alcohol dehydrogenase 1 and 4, two potent embryonic synthesizers of RA, are not expressed in the eye during RGC development (Vonesch et al., 1994; Ang et al., 1996a; Ang et al., 1996b). Thus, CYP1B1 mutations may potentially lead to congenital glaucoma by disrupting normal RA production in RGCs and subsequently impairing RGC survival during development or in the adult. It is interesting to hypothesize that CYP1B1 downregulation might also help to explain the timing of developmental cell death in RGCs.

We also found that RGCs express a number of genes found at loci linked to glaucoma. Our findings suggest a developmental function for these genes in RGCs and raise the hypothesis that cell-autonomous expression of mutated or misexpressed glaucoma-linked genes including CYP1B1 or optineurin may directly induce susceptibility in RGCs to relatively elevated intraocular pressures, leading to dysfunction and cell death in RGCs. Any of these proteins may also decrease the ability of RGCs to regenerate their axons after injury in the optic nerve (Goldberg and Barres, 2000).

**Axon injury does not recapitulate developmental gene expression**

RGCs and other CNS neurons fail to regenerate their axons in the adult CNS environment. This failure is generally attributed to a glial environment rich in axon inhibitory molecules and poor in growth-promoting trophic factors or substrates. Blocking inhibitory glial signaling and adding exogenous trophic factors, however, generally only allows a small percentage of CNS neurons to regenerate and at a very slow rate, suggesting that regenerative failure may be
intrinsic to adult CNS neurons. Consistent with this, we previously found that RGCs undergo a developmental loss of their intrinsic capacity for rapid axon growth, decreasing their rate of axon growth 10-fold in the first few days postnatally (Goldberg et al., 2002a).

An important question is whether regenerative axon growth programs recapitulate developmental axon growth programs and whether such a recapitulation would enhance regenerative response (Filbin, 2006). Although RGCs decrease their intrinsic axon growth capacity through development, correlating with a failure of regenerative response in vivo, certain treatments such as Rho inactivation, CNTF plus cAMP application, and lens injury are able to enhance the regenerative response of RGCs in vivo (Leon et al., 2000; Yin et al., 2003; Fischer et al., 2004). It is not known whether such treatments change the intrinsic capacity of RGCs to rapidly grow axons, or enhance trophic signaling or overcome inhibitory signals in the environment. When we compared the genes that change more than threefold in adult RGCs in response to axotomy plus lens injury, which stimulates a subset of RGCs to successfully regenerate their axons in vitro and in vivo (Fischer et al., 2004), we found that, although there was a preference for inverse regulation between genes regulated during development and after injury, there were no easy correlations between the two. For example, galactin-3 has been shown to promote neurite outgrowth (Pesheva et al., 1998), is downregulated in RGC development consistent with the loss of axon growth ability of RGCs, and is upregulated after axotomy consistent with an attempted regenerative response (Fischer et al., 2004), but is actually downregulated in
RGCs after lens injury plus axotomy compared with axotomy alone, despite the lens injury normally increasing RGCs regenerative success into the damaged optic nerve (Fischer et al., 2004). Thus, our data indicate that the genetic bases for regulating neurite outgrowth ability during development and regeneration after injury may share few similarities or involve separate mechanisms. These data generate a focused list of candidate genes, however, that may be shared between both situations, and such molecular hypotheses will likely have to be tested in vivo in injury models and during development to draw any strong conclusions.

In any case, our findings have important implications for understanding how retinal damage, and particularly the loss or injury of RGCs, can be repaired. The elaborate and complex program of gene expression changes that we identified during RGC development, together with our previous findings that RGCs irreversibly lose their capacity to rapidly extend axons, provide evidence that an intrinsic program of gene expression controls the elaborate timing and complexity of the developmental and morphological changes that an RGC undergoes during its development. A terminally differentiated RGC is not simply generated quickly in one step of gene expression changes from its progenitor cell, but rather it plays out a program of gene changes that extend over at least 3 weeks of its development. This complexity alone suggests that it is unlikely that, after injury, an RGC would suddenly reset to its embryonic state of gene expression and recapitulate this elaborate series of developmental events (such as axon growth, dendrite growth, synapse formation, target matching, synapse
elimination, myelination, etc.). In fact, we found that injured RGCs do not reset or replay normal developmental changes in RGC gene expression, and we found that similarity between the gene changes occurring during development and after injury was limited. Thus, to induce robust regeneration of RGCs, it may be necessary to either induce adult RGCs to revert to a newly generated transcriptional state, if this is possible, or perhaps to generate new RGCs from embryonic stem cells or retinal progenitor cells (Lamba et al., 2006).
Figure 4.1 Gene expression of RGCs through development. (A) Reproducibility of samples. Two biological replicate samples of E17 RGCs are plotted against each other, with 2-, 3-, 5-, and 10-fold difference bars included. (B) Number of genes expressed in RGCs, as defined by present calls in Microarray Suite 5.0 (Affymetrix), plotted as a histogram. (C) Of the probes present in at least 10% of RGC samples through development, the number of probe sets that change through RGC development from E17 to P21, plotted as a histogram. (D) P21 RGC versus adult whole retinal gene expression, plotting only the genes from U34A GeneChips found present in the RGC samples. Tenfold difference lines are plotted.
Figure 4.2 Validation of microarray data. (A) Comparison of fold changes (FC) in gene expression during RGC development by GeneChip (average of at least 3 samples) and real-time RT-PCR (average of 5 samples; 1 representative of 3 separate data replications are shown). (B) Lysyl oxidase protein is upregulated in rat retinal cryosections in P8 RGCs compared with E20 retinal sections in the ganglion cell layer (GCL) and the adjacent inner plexiform layer in which RGC dendrites are located (top row). A similar upregulation is seen from E20 to P8 RGC cultures (bottom row). Scale bar: top row, 450 µm; bottom row, 50 µm.
Figure 4.3 Non-negative matrix factorization. Datasets were weaned to include only genes present in at least 10% of RGC samples and changing at least threefold through development, analyzed by NMF, and internally sorted to amplify boundary edges. The axes demonstrate the ages of the RGC samples analyzed.
Figure 4.4 Hierarchical cluster analysis limited to four clusters revealed one cluster of genes that decreased perinataly, two clusters of genes that increased perinataly (only 1 is shown here), and a cluster of genes that spiked at birth and then returned to lower levels after the first postnatal week. Each graph shows the normalized expression levels through development of the highest fold-changing individual genes. Probes from these clusters are listed in supplemental Table 4. 3, available at http://www.jneurosci.org/cgi/data/27/32/8593/DC1/2.
Figure 4.5 Genes expressed by RGCs that are regulated both during development and in regeneration. (A) Venn diagram demonstrating distribution of genes analyzed. (B) Pie chart demonstrating four classes of genes regulated up or down during development and up or down during regeneration.
Figure 4.6 Expression of cochlin, CYP1B1 (CYP), and optineurin (OPTN) mRNA and protein in RGCs in vitro and in vivo. (A) Expression levels of the three mRNAs through RGC development from E17 to P21 from GeneChip data. (B) Immunoreactivity in the embryonic (E20), postnatal (P8), and early adult (P21) retina. Bright, punctuate staining in the ganglion cell layer (up in each panel) are particularly observed for CYP1B1 at E20 and for optineurin at P21. Cochlin also demonstrates an increase in expression level in the ganglion cell layer from E20 to P21, mimicking the mRNA data. (C) Western blot analysis demonstrates cochlin, CYP1B1, and optineurin expression in lysates (15 µg of protein per lane) from purified E20 and P8 RGCs. High CYP1B1 protein level is detected embryonically, whereas increased optineurin and cochlin protein levels are higher postnatally. Blotting against actin (bottom row) demonstrates similar protein loading. (D) Immunoreactivity in purified E20 and P8 RGCs after 24 h in culture. Mixed nuclear and cytoplasmic stainings were observed for cochlin (green) and optineurin (red). Perinuclear localization of endogenous CYP1B1 (red) is prominent at both ages. Scale bars, 40 µm.
Figure 4.7 Effect of glaucoma-associated genes on RGC survival in vitro. (A, B) Embryonic or postnatal RGCs as marked were transfected with plasmid controls (Ctrl) or cDNAs for CYP1B1 (CYP), optineurin (OPTN), and cochlin (COCH; performed in separate experiments and thus separated with double hash marks). RGC survival was measured 3 d later and normalized to control. Statistical analysis using SE of proportions yielded significant Z scores for CYP1B1 only (*). Representative experiments are shown. (C) Embryonic RGCs transfected with an siRNA construct directed against CYP1B1 (si-CYP) demonstrated lower survival compared with control cultures.
Supplemental figure 4.1 Relationship of “present” call to expression level. Histogram demonstrates the number of probes found to be “present,” “marginal,” or “absent” according to the Microarray Suite 5.0 algorithm according to absolute level of expression (arbitrary units).
**Table 4.1** Genes expressed at least 20-fold higher in P21 RGCs than in adult retina

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<th>Gene symbol</th>
<th>Description</th>
<th>Fold change RGCs/retina</th>
<th>References</th>
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<td>Nfl2</td>
<td>NEL-like 2 homolog (chicken)</td>
<td>261.07</td>
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<td>Mes mesorexin hypothetical LOC143461</td>
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<td>Trpaa2</td>
<td>Tropomodulin 2</td>
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<td>Lsmap</td>
<td>Limbic system-associated membrane protein</td>
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<td>clg2</td>
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<td>LOC901335</td>
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<td>Scn5a</td>
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*Predicted in reference(s) given.

*Not found in retina.

*Induced in RGCs after injury.

*May be RGC specific at specific developmental stages.
Table 4.2 Analysis of gene ontologies expressed by RGCs through development

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Bold entries denote gene ontologies statistically less likely to change; italic entries denote gene ontologies statistically more likely to change. GO, Gene ontologies; FC, fold change.

<sup>a</sup>Not transcription.