Neuronal Diversification Within the Retina:

Generation of Crossed and Uncrossed Retinal Ganglion Cells

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Abstract

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Recent advances in the field of axon guidance have revealed complex transcription factor codes that regulate neuronal subtype identity and their corresponding axon projections. Retinal axon divergence at the optic chiasm midline is key to the establishment of binocular vision in higher vertebrates. In the visual system of binocular animals, the ipsilaterally and contralaterally projecting retinal ganglion cells are distinguished by the laterality of their axonal projections. Specific axon guidance receptors and their ligands are expressed in retinal ganglion cells (RGCs) and at the chiasm, tightly regulating the development of the ipsilateral (uncrossed) and contralateral (crossed) retinal projections. Though many factors are known, their dysfunction leads to only partial misrouting of RGC axons. Moreover, the complex transcription factor codes that regulate RGC subtype identity are only beginning to be uncovered. Numerous gaps remain in our understanding of how these guidance molecules are transcriptionally regulated and how they are induced by the patterning genes that set up the different domains in which these RGC subtypes reside. An even more elusive question within the field is how the ipsilateral and contralateral RGC subpopulations acquire their different cell fates.
In this thesis, I present my work on dissecting out the molecular signatures of the ipsilateral and contralateral RGC populations during embryonic development through gene profiling followed by the functional characterization of one candidate from this screen. In Chapter 2, I developed a cell purification method based on retrograde labeling of these two cell populations from their divergent axonal projections followed by cell sorting. This method can be used in studies requiring purified populations of embryonic RGCs.

In Chapter 3, I conducted a microarray screen of purified ipsilateral and contralateral RGCs using the above method. Through subsequent validation of the in vivo expression patterns of select candidates, I identified a number of genes that are differentially expressed in ipsilateral and contralateral RGCs. Subsequent functional characterization of these genes has the potential to uncover novel mechanisms for regulating axon guidance, cell differentiation, fate specification, and other regulatory pathways in ipsilateral and contralateral RGC development and function. The results of this screen also revealed that ipsilateral and contralateral RGC may have distinct developmental origins and utilize different strategies for differentiation.

In Chapter 4, I demonstrate a novel role for cyclin D2, one of the above candidates, in the production of ipsilateral RGCs. The G1-active cyclin D2 is highly expressed in the ventral peripheral retina preceding and coincident with the developmental window of ipsilateral RGC genesis. I further found that ipsilateral RGC production is disrupted in the cyclin D2 null mouse. The expression of cyclin D2 in a distinct proliferative zone that has evolutionary significance in ipsilateral RGC production and its subtype-specific requirement during retinal development suggest that cyclin D2 may mark a distinct progenitor pool for ipsilateral RGCs. Thus, these studies offer an important advance in our understanding of neuronal subtype diversification within the retina.
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For my family.
Chapter 1. Introduction: neuronal diversification within the vertebrate retina

Introduction

The astonishing complexity of the central nervous system (CNS) has been documented in the modern era of neuroscience, starting with the late nineteenth century neuroanatomical works of Camillo Golgi and Santiago Ramón y Cajal using Golgi’s silver nitrate staining methods. Underlying this complexity is a network of highly diverse neurons that have a wide variety of morphological forms and make both stereotyped and experience-dependent connections. How this complexity is achieved within a developing organism has been an outstanding question over the last century. It is only through a growing understanding of how the mature nervous system functions, the advent of advanced techniques in molecular biology, genetics and imaging, and the establishment of simple model systems that we have begun explore this question.

The eye is especially important to the analysis of neural development because of the central role it played in the earliest studies of both cellular neuroanatomy and embryology. In addition to Ramón y Cajal’s cataloguing of neuronal classes within the retina (Cajal, 1983), Hans Spemann’s discovery of the dependence of lens development on inductive signals from the optic cup served as the foundation of his lifelong work on organizing centers in embryonic development (Spemann, 1924). Our current understanding of neurogenesis and neuronal diversification within the retina is the product of over one hundred years of convergent progress within these two fields. The vertebrate retina has been a useful model for studying general principles of neurogenesis and axon guidance due to its ease of access for visualization and
manipulation. Of particular interest to our lab are the decussating retinogeniculate projections that underlie binocular vision. The anatomical changes of axon growth cones as they exit the retina and interact with intermediate cues along their projection path and, more recently, the molecular pathways that regulate these changes have been studied in detail. However, a recent shift in focus has led many in the axon guidance field towards unraveling the upstream regulators that direct the expression of guidance programs and the mechanisms that specify the diversity of cell types that give rise to these projections. In this chapter, I will present a general overview of vertebrate retinal histogenesis and cell fate determination to introduce known mechanisms used for generating cellular diversity. I will then review the development of the binocular retinal projections, a model for studying neuronal subtype specification within the retina. Finally, I will discuss the regulatory strategies, including spatiotemporal control of neurogenesis, that have been shown to be important for neuronal subtype specification in other CNS regions and that may inform us on how subtype diversity within the retina is generated.

**Cellular diversity within the central nervous system**

First described by Karl Deiters in 1865 after painstaking surgical dissection, the neuron has a prototypical structure consisting of a cell body with protruding processes that receive (dendrites) and send (axons) information to and from partner cells (Sabbatini, 2003). Beneath this generalization, however, lies a diverse world of highly specialized neurons with varying morphology (e.g. polarity, axonal length, complexity of dendritic and axonal arbors), neurotransmitters released, and choice of cellular partners. This is manifested most clearly in regions such as the cerebellum, cerebral and hippocampal cortex, and retina.
While initial work on understanding neuronal diversity was mainly descriptive or electrophysiological, recent efforts have turned to identifying the molecular mechanisms that define each of these distinct features, both transient ones during development and persistent ones throughout the lifetime of the cell. These mechanisms will be discussed in more detail within subsequent sections of this chapter.

**Cellular diversity within the retina**

Perhaps nowhere in the vertebrate CNS is the characterization of cellular diversity as extensive as in retina, one of Ramón y Cajal’s favorite tissues (Fig. 1.1a). The retina is composed of three cellular layers that contain six classes of neurons (retinal ganglion cells or RGCs, amacrine cells, bipolar cells, horizontal cells, rods, and cones) and one class of glia (Müller cells), as well as two intervening synaptic (or plexiform) layers (Fig. 1.1b). Each class of cells can be further divided into morphologically and functionally distinct subtypes (Fig. 1.1c). Recent efforts have turned to identifying the molecular programs that set up these morphological and functional differences within neuronal classes, such as amacrine cell and RGC subtypes (Kim et al., 2008, Badea et al., 2009, Kay et al., 2011). An emerging question is how this diversity is generated during early development.

**Histogenesis of the vertebrate retina**

Unlike other sensory organs, whose most peripheral neural components are generated from migrating neural crest cells, the retina derives directly from neuroepithelium as an outpouching of the developing forebrain. Thus, insights gleaned from studies of retinal development have been influential on studies of other portions of the CNS and vice versa. As in
all neural tissue, the sequential differentiation of multipotent, naïve neuroepithelium into progressively fate-restricted progenitors and, finally, highly specialized cellular subtypes within the retina, is a process modulated by a multitude of intrinsic and extrinsic regulatory mechanisms. In this section, I will present an overview of the histological changes and known molecular pathways underlying this developmental process.

**Induction of the eye fields and optic cup development**

Following its formation, the neural tube must be further compartmentalized into specialized regions that will become the future forebrain, midbrain, hindbrain, and spinal cord. This regionalization is set up by morphogenic gradients along the anterior-posterior (A-P) axis. Members of the fibroblast growth factor (Fgf), retinoic acid (RA), Wnt, and Tgf-β families are released from caudal organizing centers to diversify the default anterior fate of neuroepithelium into more posterior fates, such as midbrain, hindbrain, and spinal cord (Altmann and Brivanlou, 2001). A number of inductive signals are now known to be important for the specification of the generic anterior neuroepithelium into the eye field (Graw, 2010). Of the signaling programs important for neural patterning, Wnt and Fgf pathways are critical for patterning of the eye field (Rasmussen et al., 2001, Moore et al., 2004, Cavodeassi et al., 2005).

Collectively, these diverse signaling pathways lead to the induction of a network of evolutionarily conserved transcription factors in the eye field upon its specification. This group of “eye field transcription factors” (EFTFs) include a number of homeobox genes, such as Rax, Pax6, Six3, Six6, and Lhx2 (Fuhrmann, 2010). In one example, the *Rax* null mouse mutant fails to develop optic cups (Mathers et al., 1997). The spontaneous *eyeless* mouse mutant, first studied in the 1940s, was since found to carry a point mutation in *Rax* (Chase, 1944, Tucker et
Moreover, mutations of *Rax* homologs in zebrafish and humans similarly lead to failure of eye formation (Loosli et al., 2003, Lequeux et al., 2008). While loss of other EFTFs leads to microphthalmia or anophthalmia, Rax appears to act upstream of its fellow EFTFs. For example, Rax expression is normal in Pax6−/− mice but Pax6 expression is lost in Rax−/− mice (Zhang et al., 2000). Many of these EFTFs have also been shown to play additional roles in retinal development, such as promoting continued proliferation of retinal progenitor cells (RPCs) prior to the onset of differentiation (Zuber et al., 1999, Casarosa et al., 2003, Miyawaki et al., 2004, Zaghloul and Moody, 2007, Tétreault et al., 2009).

Following its induction, the anterior eye field must separate into the left and right eye primordia in a process driven by Nodal and Sonic hedgehog (Shh) signaling from the developing dorsoventral axis of the embryo (Fuhrmann, 2010). These signaling events are thought to repress Pax6 and reprogram midline eye field cells into ventral diencephalic fates (Müller et al., 2000). Indeed, Shh mutations in both animal models and humans have been associated with holoprosencephaly and cyclopia (Chiang et al., 1996). At this stage, the neuroepithelium that will give rise to the future retinal structures has been molecularly patterned but remains morphologically identical to its neighboring regions.

Eye morphogenesis begins with the formation of shallow pits, known as optic sulci, at the anterior neural tube prior to its complete closure. The optic sulci next evaginate from the developing forebrain to form the hollow optic vesicles that are in close juxtaposition with the surface ectoderm. Through a series of inductive events, the surface ectoderm thickens and invaginates to become the lens pit whereas the optic vesicle folds inwards to form a two-layered cup, with the distal/ventral domain of the optic vesicle developing into the neural retina and the dorsal into the retinal pigment epithelium (RPE) (Fig. 1.2). The optic cup remains connected to
the diencephalon through the optic stalk, which forms the scaffold for the future retinal axons that coalesce to form the optic nerve. A combination of extracellular signals from the extraocular mesenchyme and lens ectoderm activate a transcriptional network within the optic cup that culminates in the domain-specific expression of homeobox protein Vsx2 (formerly Chx10) in the neural retina and bHLH transcription factor Mitf in the RPE (Fuhrmann, 2010).

Optic cup morphogenesis is now known to be a self-organizing process, as Yoshiki Sasai’s group beautifully showed in a three-dimensional embryonic stem cell (ESC) culture system (Eiraku et al., 2011). ESC-derived neurospheres spontaneously form Rax-expression domains regions that subsequently undergo the same morphogenic changes and gene expression progression as the developing optic cup in vivo. Amazingly, these in vitro optic cups form both RPE and neuroepithelium that exhibits interkinetic nuclear migration, forming the fully stratified layers of the neural retina. In vivo, however, the surface-ectoderm derived lens and neural crest-derived mesenchyme surrounding the retina are an important source of molecular cues that influence retinal morphogenesis and patterning (Fuhrmann, 2010).

**Regulation of retinal progenitor competence**

Neurogenesis commences within the inner layer of the optic cup following the patterning events that distinguish neural retina from RPE, with growth of the retina occuring through a series of symmetric and asymmetric cell divisions that expand the progenitor pool and give rise to the differentiated cell types of the three-layered retina (Chow and Lang, 2001). Differentiation follows a central to peripheral pattern in most vertebrate species. Fgf signaling from the optic stalk and central retina is thought to initiate this wave of differentiation (Esteve and Bovolenta, 2006).
Clonal analysis with retroviral vectors induced in a small number of dividing cells showed that a radial cluster formed from a common progenitor can contain multiple neuronal cell types and glia (Turner and Cepko, 1987). Early birthdating studies with [3H]thymidine revealed that, like in cortex (Frantz and McConnell, 1996), cell types residing in different retinal laminae are generated in a characteristic order, grouped in two phases: 1) RGCs, horizontal cells, cones, and amacrine cells; 2) bipolar cells, rods, and finally Müller glia (Cepko et al., 1996, Hu and Easter, 1999). RPCs are thus thought to move through a series of intrinsically determined competence states during which the seven different cells classes are generated (Fig. 1.3) (Livesey and Cepko, 2001, Cayouette et al., 2006).

The regulation of temporal competence within the CNS can be generalized into two different mechanisms (Pearson and Doe, 2004). In the cortex and spinal cord, temporal identity is thought to be regulated extrinsically, where progenitor cells respond to patterning cues that change over time, leading to changes in the identity of their progeny (Pearson and Doe, 2004, Caviness et al., 2009). In the retina, however, temporal identity appears to be regulated in a similar manner as seen with Drosophila neuroblasts, where progenitors acquire different initial identities based on spatial patterning cues but then produce lineages strictly based on birth order, independent of further environmental changes (Dyer and Cepko, 2001, Pearson and Doe, 2004). Consistent with this model, retinal progenitors are not synchronized, and retinal neuron classes are generated in overlapping and heterogeneous waves. In contrast, the temporal (birth-date) and laminar identity of cortical neurons are tightly correlated, suggesting that a global timing cue directs cortical progenitors to produce neurons for each lamina in synchrony (Pearson and Doe, 2004, Caviness et al., 2009).
Evidence supporting cell-intrinsic mechanisms in the regulation of RPC competence came from fate-mapping experiments, in which differentiation of cultured RPCs into postmitotic cell types is dependent on the age of retina from which they are extracted (Reh and Kljavin, 1989, Watanabe and Raff, 1990). These studies were followed up by heterochronic culture experiments in which rodent or chick progenitors from different stages of development were cultured in an earlier or later environment. In both conditions, progenitors mostly give rise to cell types appropriate for their developmental state rather than that of their environment. (Austin et al., 1995, Morrow et al., 1998, Belliveau and Cepko, 1999, Belliveau et al., 2000, Rapaport et al., 2001). There is, however, a degree of flexibility within fate determination that can be influenced by the external environment, especially in the progeny of earlier RPCs (Reh and Kljavin, 1989, Belliveau and Cepko, 1999). The loss in ability to generate earlier cell types (e.g RGCs and amacrine) by embryonic progenitors co-cultured with older cell types is likely due to feedback mechanisms from differentiated RGCs and amacrine cells in culture (Waid and McLoon, 1998, Belliveau and Cepko, 1999). Late progenitors show more restrictions in the cell types they can produce, as re-aggregate culture systems in which postnatal progenitors are mixed with embryonic retinal cells show an increased bias towards producing earlier cell types (bipolar) but only within their normal lineage and with the same kinetics (Belliveau et al., 2000). That is, although the relative contribution of the different progeny classes can change, the type of progeny that can be generated does not.

Thus, while extrinsic factors may alter the number of cells produced within each competence state and, hence, the balance of cellular composition within the retina, intrinsic mechanisms drive the progressive restriction in retinal progenitor competence. In Drosophila, the expression of zinc finger transcription factor hunchback specifies progenitor competence for
early cell fates (Pearson and Doe, 2004). Similarly, its mouse ortholog Ikaros is expressed in early RPCs, and misexpression of Ikaros in late RPCs leads them to produce early-born neurons, thereby conferring early temporal competence to these RPCs.

**Generating diversity: mechanisms for cell fate specification in the retina**

While the temporal transition in retinal progenitor competence sets the time course for cell type production, fate determination of resulting progeny occurs under the direction of a number of intrinsic and extrinsic regulators acting upon the postmitotic cell or even upon their progenitors. The culmination of these regulatory mechanisms is the generation of the proper cell types in the proper ratios within the retina. Here, I will discuss some of the known mechanisms through which neuronal diversity is generated within the retina.

**Intrinsic mechanisms**

In addition to their lack of synchronization, RPCs are heterogeneous in the number of divisions they undergo and the size and cell compositions of the clones they make (Wetts and Fraser, 1988, Agathocleous and Harris, 2009). Newer genomic techniques have further revealed an incredible degree of spatiotemporal heterogeneity in molecular identity (Blackshaw et al., 2004, Trimarchi et al., 2008b, Riesenberg et al., 2009). Manipulation of proneural genes, particularly the bHLH class of transcription factors, can alter both the balance of retinal cell fates and the timing of cell cycle exit. One example is Math5 (Ath5 in zebrafish and Xath5 in *Xenopus*), which is expressed during the terminal G2 cell cycle phase of retinal progenitor cells and biases them to differentiate into retinal ganglion cells (Poggi et al., 2005). Transcription
factor FoxN4 is also expressed in a spatially restricted pool of RPCs, and loss of FoxN4 leads to failure of horizontal cell production and a decrease in amacrine cell production (Li et al., 2004).

Many of the early EFTFs have been shown to activate cell cycle progression directly (Green et al., 2003) in addition to having other functions important for the generation of retinal cell types. One such EFTF, Pax6, is required for the expression of various bHLH transcription factors within the retina (Marquardt et al., 2001) and can directly induce Ath5 expression (Willardsen et al., 2009).

**Extrinsic signaling**

Extrinsic signals can influence both the onset and rate of proliferation, cell cycle exit, and fate decisions. Many of the same signaling pathways appear to play multiple roles at different developmental timepoints. Shh signaling is unquestionably important for RGC production, but studies that have tried to tease out its role have often come to conflicting conclusions. In zebrafish, Shh expression by newly differentiated RGCs induces neighboring cells to exit cell cycle and differentiate into RGCs, thus initiating a central to peripheral wave of RGC differentiation. Disruption of Shh signaling during neurogenesis blocks RGC differentiation (Neumann and Nuesslein-Volhard, 2000) and reduces cell cycle inhibitor p27Kip2, leading to continued proliferation of progenitors (Shkumatava and Neumann, 2005). Studies conducted in chick and mouse, on the other hand, have shown that Shh release by differentiated RGCs serves as a negative feedback signal to retinal progenitors to stop production of additional RGCs (Zhang and Yang, 2001). Ablation of Shh in murine retina leads to precocious cell cycle exit and a depletion of progenitor cells, leading to the overproduction of RGCs at the expense of later-born cell types, such as bipolar cells and Muller glia (Wang et al., 2005). Conversely, when RGCs are
lost in the Brn3b\(^{-/-}\) mouse, Shh activity is decreased and RPC proliferation decreases (Mu and Klein, 2004).

While these conflicting results may reflect species-specific differences in Shh function, a better understanding of the mechanisms of Shh signaling has the potential of reconciling these results. More recent studies in *Xenopus* and zebrafish retina have shown that Shh speeds up cell cycle, possibly through upregulation of G1 and G2 cyclins, thereby stimulating proliferation as seen in mouse and chick (Locker et al., 2006). On the other hand, Shh also leads to earlier cell cycle exit and differentiation. Thus, a new model was proposed in which in which Shh signaling induces the transition of slow-cycling, immature retinoblasts into faster-cycling RPCs that are closer to cell cycle exit and ready to differentiate into postmitotic neurons (Agathocleous et al., 2007, Agathocleous and Harris, 2009). Although this model does not explain the role of Shh in controlling RGC number through negative feedback, it does link the signaling pathway to cell cycle regulation within RPCs, suggesting that cell cycle control may contribute to RPC competence transitions.

**Cell cycle regulation and fate determination**

Cell cycle regulation is a powerful mechanism for controlling the cellular makeup of a region, and changes in cell cycle progression or exit can dramatically change the balance of cell types within the retina. Cell cycle kinetics also change during retinal development, such that late progenitors take longer to divide (Alexiades and Cepko, 1996). Studies in *Xenopus* suggested that longer cell cycle length allows for the translation and accumulation of homeobox genes such as Otx5b, Vsx1, and Otx2, which promote later bipolar and photoreceptor cell fates (Decembrini et al., 2006). The expression of cell cycle regulators is also homogeneous among progenitors,
suggesting that the proliferation requirements of progenitors change over time. For example, of the G1 cyclins, Cyclin D1 is important for cell cycle progression throughout retinal development, but Cyclin D3 is not expressed until the end of histogenesis and cannot drive proliferation at earlier stages of development (Donovan and Dyer, 2005). I will expand upon the discussion of G1 cyclins later in the chapter in the context of fate determination of neuronal subtypes.

**The emergence of retinal neuronal subtypes**

The cumulative work of many groups over the last two decades have elucidated many of the mechanisms used in generating neuronal diversity throughout the retina and other parts of the nervous system. Most of this research, and my discussion thus far, has centered on the production of broad classes of neurons or glia. A growing effort in recent years has moved towards uncovering the mechanisms behind the further diversification of neuronal subtypes. In the next section, I will discuss our current understanding of two specific subtypes within the retina, the ipsilaterally and contralaterally projecting RGCs.

**Development of the binocular retinal projections**

This section is adapted from a book chapter I co-authored for *The New Visual Sciences* (Mason, Kuwajima, and Wang, 2014).

In most lower vertebrates, the lateral positioning of the eyes results in each eye receiving completely non-overlapping visual information. Binocularity is a feature found in higher vertebrate species that have more frontally positioned eyes, which allows for convergence of the bilateral visual fields. As a result, information from the same part of the visual field is perceived
by both eyes but in a slightly different way. This conflict is capitalized by higher order visual processing for depth perception, or stereopsis, thus allowing for depth of field at the expense of breadth.

In all vertebrates, the axons of retinal ganglion cells (RGCs) involved in visual information processing grow out from each eye toward the brain and meet at the midline in the ventral diencephalon, where they establish an X-shaped pathway, known as the optic chiasm, and then extend to targets in the thalamus (dorsal lateral geniculate nucleus; dLGN) and the midbrain (superior colliculus; SC; or optic tectum in lower vertebrates). The patterns of RGC axon projection at the optic chiasm in different species range from complete crossing, with segregation of fibers from each eye, to partial decussation, with complex intermingling of the fibers from the two eyes. The presence and relative size of the ipsilateral projection depends on the degree of binocular overlap in the visual field (Fig. 1.4). In lower vertebrates that lack binocular vision, such as fish, the fibers from each eye form an entirely crossed projection. The tadpoles of some amphibian species, such as *Xenopus laevis*, also have an entirely crossed projection, but an ipsilateral projection develops during metamorphosis when the eyes change position and binocularity develops (Hoskins and Grobstein, 1985a, Mann and Holt, 2001). Adult chickens have an entirely crossed projection, but in early development there is a transient ipsilateral projection (O'Leary et al., 1983, Thanos and Bonhoeffer, 1984).

In mammals, partial decussation occurs, and the degree of crossing varies widely among species. In humans and primates, all RGC axons originating from the nasal retina cross the midline and project into the contralateral optic tract, and all RGC axons from the temporal retina project into the ipsilateral optic tract, resulting in ~40% of RGC axons projecting ipsilaterally (Polyak, 1957, Chalupa and Lia, 1991). In ferrets and cats ~12%–15% of RGCs project
ipsilaterally. In mice, however, only a small proportion of RGCs (<5%) located in the peripheral ventrotemporal (VT) retina project ipsilaterally, and these cells are intermingled with contralaterally projecting RGCs (Guillery et al., 1995, Jeffery, 2001).

\textit{Time course of retinal axon outgrowth and divergence}

Studies in the past 30 years have characterized the birthdates of ipsilateral and contralateral RGCs and the timing of axon growth through the optic chiasm (Dräger, 1985, Colello and Guillery, 1990, Sretavan, 1990). The different subpopulations of RGCs, from distinct retina regions, are produced in discrete periods. RGC axon outgrowth in the mouse takes place at three discrete stages of optic chiasm that lead to corresponding to the laterality of these projections (Fig. 1.5). From embryonic day (E) 12 to E13, the earliest-born RGCs originating from dorsocentral (DC) retina extend through the optic stalk and along the pia of the ventral diencephalon both contralaterally and ipsilaterally (Colello and Guillery, 1990, Marcus and Mason, 1995). These early uncrossed axons grow directly into the ipsilateral optic tract without encountering the midline and are thought to pioneer the retinal axon pathway as seen in zebrafish (Pittman et al., 2008). However, the fate of these early-born ipsilaterally projecting RGCs is unknown as they cannot be retrogradely labeled in adulthood. Thus it remains to be determined whether they die, retract their ipsilateral projections, or migrate to the VT retina.

During the peak phase of retinal axon divergence from E14 to E17, RGC differentiation expands from the DC region to the peripheral circumference of the retina. The permanent ipsilateral projection emerges from RGCs in the most peripheral VT retinal crescent, and the contralateral projection forms from RGCs outside of the VT crescent. However, during the late phase of RGC axon extension from E17 to postnatal day (P) 0, newborn RGCs in the VT
crescent project contralaterally rather than ipsilaterally. Thus, from E14 to E17, RGCs from the ventrotemporal retina project primarily to ipsilateral targets, but after E17 the last-born RGCs within VT retina project contralaterally. Therefore, it appears that ipsilateral RGCs are specified within a tight spatiotemporal window that is superimposed on the normal progression RGC differentiation within the retina.

**Molecular guidance programs that direct retinal ganglion cell axon divergence**

During optic chiasm formation, the growing tips, or growth cones, of both crossed and uncrossed RGC axons enter the chiasm region. Unlike the early-born ipsilateral projection arising from dorsocentral retina that diverge from the contralateral projection at the lateral optic chiasm, the axons that give rise to the permanent ipsilateral projection and that originate in VT retina separate from axons projecting contralaterally close to the midline (Godement et al., 1990, Godement et al., 1994). Live imaging of growth cone behaviors has revealed that although both uncrossed and crossed growth cones pause at the midline and undergo extension–retraction behavior, uncrossed axons pause for longer periods and form Y-shaped or multiple growth cones as compared to crossed axons, which bear individual, more simple growth cones (Godement et al., 1994). These observations suggested that RGC growth cones sense and respond to guidance cues within the cell populations around the midline of the optic chiasm.

Analysis of retina–chiasm cocultures showed that the chiasmatic neurons and glia provide such cues for retinal axon growth and divergence. When grown with chiasm explants in collagen gel cultures, RGCs from all retinal regions display reduced neurite outgrowth (Wang et al., 1996), indicating that chiasm cells express diffusible inhibitory cues to retinal axons. However, when grown directly on dissociated chiasm cells in two-dimensional cultures, VT
explants show a 60% reduction in neurite outgrowth whereas extra-VT (e.g. dorsotemporal or DT) retinal explants show only a 20% reduction (Wang et al., 1995). Thus, while the chiasm appears to be somewhat inhibitory to all RGC axon growth, it inhibits ipsilateral RGC axon growth to a greater extent, reflecting the differential behavior of axons of these two populations of RGCs at the midline.

These early studies suggested that multiple guidance cues presented by chiasm cells underlie the differential response of the axons of crossed and uncrossed RGCs through both diffusible and contact-mediated interactions. However, the molecular identity of these cues was unknown until the last decade. The first guidance cue that was implicated in retinal axon divergence at the optic chiasm, Ephrin-B, is expressed at the chiasm of metamorphic but not premetamorphic *Xenopus* and repels EphB-expressing RGCs during the formation of the ipsilateral projection. Precocious expression of ephrin-B in the tadpole chiasm is sufficient for inducing an ipsilateral projection from EphB+ RGCs. Ephrin-Bs are also expressed in the chiasm of mammals with binocular vision but not in the chiasm of fish and birds with monocular vision (Nakagawa et al., 2000).

Detailed analysis of the mouse optic chiasm revealed that Ephrin-B2 is expressed by radial glial during the peak phase of RGC axon outgrowth when the permanent ipsilateral projection develops. Moreover, ephrinB2 specifically inhibits outgrowth of uncrossed RGC axons, and blocking ephrinB2 relieves this inhibition *in vitro*. EphB1, a receptor for ephrinB2, is expressed in regions of retina that give rise to the ipsilateral projection during the same window of expression as that of ephrinB2 at the chiasm midline. *EphB1*−/− mutants display a reduced ipsilateral projection (Williams et al., 2003). Ectopic EphB1 expression in extra-VT retina redirects RGCs ipsilaterally, an effect that requires both the extracellular and juxtamembrane
domains of EphB1 (Petros et al., 2009c). Together, these data suggest that the function of B-class Ephs and ephrins in patterning binocular vision is conserved between species, and the inhibitory EphB1-ephrinB2 interaction drives VT axons to project ipsilaterally at the mouse optic chiasm midline.

The protean Shh signaling pathway has also been implicated in chiasm crossing (Trousse et al., 2001, Sánchez-Camacho and Bovolenta, 2008). Ipsilateral RGC axons that express the Shh receptor Boc retract in the presence of Shh, while Boc\(^{-/-}\) axons show no response to Shh \textit{in vitro}. Moreover, Boc\(^{-/-}\) mice have a decreased ipsilateral projection, and ectopic expression of Boc in contralateral RGCs causes axons to project ipsilaterally (Fabre et al., 2010). Although these results implicate an inhibitory interaction between Boc and Shh in the formation of the ipsilateral projection, whether Boc directs RGC axons as a guidance receptor for chiasm Shh or instead influences ipsilateral RGC specification within the retina remains to be determined.

After the discovery of ipsilateral guidance programs, efforts turned to determining whether midline crossing at the optic chiasm involves attractive or growth-supporting cues, similar to the role of netrin in the ventral midline of the mouse spinal cord (Dickson and Gilestro, 2006). However, such signals have not been identified at the optic chiasm until recently (Erskine et al., 2011). Netrin-1 itself is expressed at the optic disc rather than at the chiasm midline (Deiner et al., 1997), and accordingly, in mutants of netrin-1 or its receptor, deleted in colon cancer (DCC), axons fail to enter the optic nerve while retinal decussation at the optic chiasm remains normal (Deiner and Sretavan, 1999).

The semaphorins, a large family of axon guidance cues that usually play a repulsive role, have surprisingly been reported to mediate midline crossing of RGC axons at the optic chiasm
and commissural axons in the spinal cord (Nawabi and Castellani, 2011). Neuropilin-1, a receptor for the Sema3 subfamily, is expressed in crossed RGCs during development (Erskine et al., 2011). VEGF164, the neuropilin-binding isoform of classical VEGF, is expressed at the chiasm midline from E12 to E17. Intriguingly, VEGF164 acts as an attractive diffusible cue that promotes axon outgrowth and attracts growth cones of neuropilin-1+ RGCs in vitro. Mutants lacking Neuropilin1 or VEGF164 display an increased ipsilateral projection and highly defasciculated RGC axons in the chiasm. Thus, neuropilin and VEGF interactions are thought to facilitate the formation of the contralateral projection by providing permissive signals to RGC axons.

NrCAM, a member of the immunoglobulin (Ig) superfamily of cell adhesion molecules, is also expressed in crossed RGCs throughout the retina during all phases of axon outgrowth (Lustig et al., 2001, Williams et al., 2006a). Blocking NrCAM function leads to decreased axon outgrowth of crossed RGCs cocultured with dissociated chiasm cells and to an increased ipsilateral projection in semi-intact visual system preparations. NrCAM−/− mice similarly display an increased ipsilateral projection in vivo, but these fibers arise only from the late-born VT RGCs (Williams et al., 2006a). Thus, NrCAM is necessary for the contralateral projection at the later age of chiasm formation.

As midline zones contain both inhibitory and growth-supporting cues (Nawabi et al., 2010, Parra and Zou, 2010), attraction is not the only mechanism that can implement midline crossing. Supporting this hypothesis, contralateral RGC growth cones spend several hours advancing and retracting at the midline before rapidly crossing the midline in vivo, as though abrogation of an inhibitory cue occurs. In zebrafish, which have a completely crossed retinal projection, the repulsive guidance cue Sema3d is expressed at the midline of the ventral
diencephalon (Sakai and Halloran, 2006). Both ubiquitous Sema3d overexpression and Sema3d knockdown exclusively at the chiasm midline drive axons to project ipsilaterally. These data imply that inhibitory signals, such as Sema3d, are released by the midline. Three hypotheses can explain how repulsive guidance cues mediate midline crossing: (1) inhibitory midline signals are converted to attractive signals; (2) inhibitory signals are diminished by degradation or cleavage of their receptors or (3) inhibitory cues are offset by other attractive signals.

Our lab has recently shown that transmembrane protein Sema6D is expressed by radial glial cells in the mouse optic chiasm along with NrCAM (Kuwajima et al., 2012). Plexin-A1, a known receptor for Sema6D, is expressed by SSEA-1+ chiasm neurons. NrCAM and Plexin-A1 are coexpressed by RGCs that cross the midline. In vitro, Sema6D alone is inhibitory only to crossed RGCs, but the presence of both NrCAM and Plexin-A1 on chiasm cells can convert the repulsive effects of Sema6D to growth-promotion. Binding studies explain this result by demonstrating that NrCAM is a receptor for Sema6D and interacts with the Sema6D-Plexin-A1 complex. Finally, in vivo analysis of Sema6D−/− and Plexin-A1−/−;NrCAM−/− mutants indicates that NrCAM and Plexin-A1 expression is required in both RGCs and chiasm cells, along with chiasm Sema6D, for efficient decussation and fasciculation of retinal axons at the optic chiasm midline.

Although the molecular programs described above appear to be important for axon pathfinding at the chiasm, as shown by severe chiasm perturbations in mice with mutations in the guidance factors discussed above, the majority of crossed axons still cross the midline (Erskine et al., 2011, Kuwajima et al., 2012). Thus, multiple signals from different guidance families, including many not yet identified, must act together to mediate midline crossing and axonal organization at the optic chiasm.
Axon Fasciculation and Midline Crossing

Axon–axon fasciculation is critical to axon guidance, including at the midline of the neuraxis (Myers and Bastiani, 1993, Bak and Fraser, 2003, Moon and Gomez, 2005, Raper and Mason, 2010). In fish, fibers from each optic nerve are bundled, and these two bundles overlap to form the chiasm. However, in higher vertebrates, increasingly finer degrees of eye-specific bundles interweave at the chiasmatic crossing (Guillery et al., 1995).

Several molecules have been identified that might function in fasciculation generally and in retinal axon decussation at the chiasm. For example, Sema3d modulates levels of the cell adhesion molecule L1 to mediate axon–axon interactions in the zebrafish spinal cord (Wolman et al., 2004). Members of the Slit family of guidance cues, Slit1 and Slit2, are expressed in domains surrounding the path of growing retinal axons and around the chiasm midline while their receptor Robo2 is expressed in RGCs (Erskine et al., 2000). Both Slit1 and Slit2 inhibit outgrowth of all RGC axons in vitro. While neither the Slit1^−/− nor Slit2^−/− single mutant displays severe axon guidance defects, retinal axons of Slit1^−/−;Slit2^−/− double mutant mice project into the contralateral optic nerve and extend ectopically dorsal or lateral to the chiasm (Plump et al., 2002). Similar to Slit1^−/−;Slit2^−/− double mutants, Robo2^−/− mice also show an expansion of the chiasm along the rostrocaudal axis (Plachez et al., 2008). Slits are thus thought to establish a repulsive corridor to channel retinal axons and encourage fasciculation but are not critical role for axon divergence at the chiasm.

Other molecules that mediate axon fasciculation at the chiasm include heparan sulfate (HSPGs) and chondroitin sulfate proteoglycans, which are important for cell surface interactions (Reese et al., 1997, Leung et al., 2003). The HSPG modifying enzymes Hs2st and Hs6st1 are
expressed by RGCs and cells within the ventral diencephalon (Pratt et al., 2006). $Hs2st^{-/-}$ and $Hs6st1^{-/-}$ mutants display axon misrouting and defasciculation at the chiasm; however, like Slit mutants, neither mutant displays defects in midline decussation. Thus, mechanisms regulating axonal fasciculation and organization at the optic chiasm may be independent of those directing RGC divergence. Recent studies have turned to understanding other cell adhesion molecules and mediators of axon fasciculation by eye-specific cohorts.

**Projection of ipsilateral and contralateral RGCs to the dorsal lateral geniculate nucleus**

The partial decussation of RGC axons at the optic chiasm midline ensures that targets on each side of the brain receive inputs from both eyes. Within the dLGN, RGC afferents from each eye are segregated into distinct territories. In mice, the retina maps topographically onto the dLGN, directed by guidance factors and receptors in the Eph/ephrin A family (Huberman et al., 2005, Pfeiffenberger et al., 2005). In addition, retinal inputs from both eyes segregate into distinct subregions within the dLGN. Rather than adopting the laminar organization found in higher vertebrates, mouse ipsilateral fibers from VT retina form a core surrounded by contralateral inputs (Godement et al., 1984, Jaubert-Miazza et al., 2005). In contrast, the late-projecting contralateral inputs from VT retina innervate the dorsal tip of the dLGN (Pfeiffenberger et al., 2005). This precise organization of eye-specific inputs is dependent on both molecular factors and neural activity (Huberman et al., 2008).

Although the mouse dLGN has been widely used as a model for activity-dependent refinement, we are only beginning to understand the developmental progression of RGC afferent targeting in the dLGN and the molecular mechanisms directing these processes. The overall time course of projection and refinement has been outlined (Jaubert-Miazza et al., 2005, Dhande...
et al., 2011), yet the precise differences between timing and point of entry for ipsilateral versus contralateral inputs, as well as the extent to which these two cohorts are segregated in the optic tract, are not well understood.

The identification of eye-specific markers within the corresponding recipient zones of the dLGN has lagged behind an understanding of the molecules that mediate topographic connections (Huberman et al., 2008). In ferret, genes have been annotated in eye-specific layers within the dLGN (Kawasaki et al., 2004), but only after innervation is established. The best candidate thus far for ipsilateral retinogeniculate targeting is Ten_m3, an adhesion molecule in the teneurin family. Ten_m3 is expressed in the ventral retina, and Ten_m3⁻/⁻ mice show targeting defects of only ipsilateral retinal axons in the dLGN, producing deficits in binocular vision without affecting midline guidance (Leamey et al., 2007). Ongoing gene profiling efforts by multiple groups will likely identify molecules in both retina and dLGN that mediate eye-specific innervation of visual targets.

The segregation of ipsilateral and contralateral inputs from an initially overlapping pattern is an activity-dependent process (Huberman et al., 2008). Neural activity in the form of “waves” generated by amacrine and retinal ganglion cells has been implicated in the patterning of dLGN connectivity (Shatz, 1996, Pfeiffenberger et al., 2005, Torborg et al., 2005), but the precise aspects of neural activity that act on eye-specific innervation and refinement have long been under debate (Chalupa, 2009, Feller, 2009). This issue has been explored more precisely in two recent studies. In the first, Xu et al. demonstrated that wave size, rather than the presence or pattern of activity, drives eye-specific segregation (Xu et al., 2011). In another study, Ullian et al. selectively reduced glutamate release from ipsilateral-projecting RGCs and showed that glutamatergic transmission plays a role in excluding competing axons from inappropriate target
regions during visual circuit refinement but not in consolidation or maintenance of ipsilateral axonal territory, implicating other molecular mechanisms for such processes (Koch et al., 2011). However, the role neural activity plays in expression of molecules important for eye-specific targeting and segregation has not been examined.

**Transcriptional control of retinal axon decussation**

A major question in the field of axon guidance is how transcription factors that specify cell identity relate to the guidance programs that these cell types employ. Studies in the cortex, spinal cord, and retina have revealed transcription factor codes that regulate neuronal subtype identity and their corresponding axon projections (Butler and Tear, 2007, Polleux et al., 2007). In the spinal cord, combinatorial codes of LIM homeobox transcription factors within spinal levels and Hox transcription factors along the rostrocaudal axis encode the identity of distinct motor neuron pools and their projections to different muscle groups (Briscoe et al., 2000, Dasen et al., 2005). LIM homeodomain proteins have been further shown to pattern these projections by regulating the topographic expression of Eph receptors and ephrins in motor neurons and limb targets (Kania and Jessell, 2003, Luria et al., 2008, Palmesino et al., 2010). In the cortex, a network of transcription factors regulates fate determination in subpopulations of projection neurons (Molyneaux et al., 2007, Leone et al., 2008). Whether these transcription factors also regulate the expression of guidance receptors as they do in motor neurons remains to be determined.

In the visual system, a number of transcription factors regulate the topographic patterning of the retina and RGC axon targets, such as chick Vax, mouse Vax2 (Schulte et al., 1999), and *Xenopus* Tbx5 (Koshiba-Takeuchi et al., 2000), which are involved in dorsoventral patterning of
the retina. More specifically, Zic4 expression in the dLGN plays a role in the retinotopic targeting of ipsilateral RGC fibers (Horng et al., 2009) but not in the segregation of ipsilateral and contralateral RGC afferents. Although transcription factor Brn3b regulates retinal axon pathfinding at multiple points along the projection pathway (Erkman et al., 2000), none of these transcription factors appear to direct decussation or targeting of ipsilateral and contralateral RGCs.

The first discovery of a transcriptional regulator of midline decussation in the vertebrate nervous system was Zic2. The Zic family of zinc finger transcription factors (Zic1–5) is critical for early neural patterning and midline formation in the developing embryo (Nagai et al., 1997, Merzdorf, 2007), and Zic2 mutations have been implicated in human developmental defects such as holoprosencephaly (Brown et al., 1998). Zic2 is expressed early in the optic vesicle and stalk during eyecup formation but is later downregulated (Nagai et al., 1997). During the peak phase of retinal outgrowth (E14 to E17), Zic2 is again upregulated but only in RGCs of the peripheral VT crescent from which the ipsilateral retinal axons arise, indicating a tight spatiotemporal restriction of Zic2 to ipsilaterally projecting RGCs. In fact, retinal Zic2 expression tightly correlates with the size of the ipsilateral projection within different vertebrate species, as it is absent from species lacking binocular vision such as chick and zebrafish and, like EphB1, is upregulated during metamorphosis in *Xenopus* when the ipsilateral projection forms. Zic2 hypomorph mice have a severely reduced ipsilateral projection, and cultured DT retinal axons overexpressing Zic2 respond to the growth inhibitory cues of cocultured chiasm cells, similar to VT axons (Herrera et al., 2003a). Together, these experiments demonstrate that a single transcription factor is necessary and sufficient for determining laterality of RGC projections.
Interestingly, both Zic2 and EphB1 are important for RGC axon divergence at the chiasm midline and share a similarly restricted pattern of expression to ipsilaterally projecting RGCs in the VT retina. Further studies then showed that EphB1 expression is lost in Zic2 hypomorph mice, and Zic2 overexpression in extra-VT RGCs is sufficient for eliciting midline avoidance and repulsion by Ephrin-B2, mostly through EphB1-dependent mechanisms (García-Frigola et al., 2008, Lee et al., 2008). However, Zic2 overexpression is more efficient than EphB1 in inducing this ectopic ipsilateral projection (Petros et al., 2009b), and Zic2 overexpression can induce a small ipsilateral projection even in EphB1−/− mice (García-Frigola et al., 2008). Moreover, Zic2 has not been shown to directly regulate EphB1 transcription. These findings on Zic2 and EphB1 point to EphB1-independent ipsilateral guidance programs operating in the retina and also to additional transcription factors that regulate these programs.

The studies characterizing the role of Zic2 in VT retina lead to the question of whether a parallel transcriptional regulator exists for the contralateral projection. The LIM homeodomain transcription factor Islet 2 (Isl2) is expressed exclusively in contralaterally projecting RGCs during retinal development. In the VT retina, Isl2 is expressed in a non-overlapping pattern with Zic2 and is upregulated in late-born RGCs that project contralaterally. As the ipsilateral projection is increased in Isl2−/− mice, Isl2 seemed a good candidate for a Zic2-equivalent in specifying contralaterality. However, the additional ipsilaterally projecting axons in the Isl2−/− mice originate only from VT retina; moreover, Zic2 expression is upregulated in additional cells within the VT but does not expand into the extra-VT domain (Pak et al., 2004). Thus, similar to the NrCAM−/− phenotype, loss of Isl2 appears to only convert the laterality of late-born, contralaterally projecting RGCs within the VT retina.
The above studies support a model in which Isl2 specifies RGC laterality by repressing an ipsilateral guidance program involving Zic2 and EphB1 that is unique to the VT retina. However, it remains to be determined whether Isl2 indeed represses Zic2 and/or EphB1, whether this genetic interaction is through direct transcriptional regulation, and whether Zic2 similarly represses contralateral identity through repressing Isl2 and downstream genes. Studies in the spinal cord have demonstrated that cell specification can be regulated by cross-repression between spatially restricted transcription factors (Dasen, 2009, Sürmeli et al., 2011). Such cross-regulatory networks may similarly act in the retina to restrict uncrossed RGCs to the VT crescent.

As Isl2 appears to be necessary for laterality decisions only within late-born VT RGCs and is expressed in only one-third of all contralaterally projecting RGCs (Pak et al., 2004), other transcription factors are likely the important determinants of laterality in the extra-VT retinal domain. The POU-domain transcription factor Brn3a (Pou4f1) is also expressed in only contralaterally projecting RGCs; however, unlike Isl2, Brn3a−/− mutants show no defects in RGC midline crossing or in expression of genes controlling laterality (Quina et al., 2005).

Thus far, a genetic program directing the major contralateral RGC projection from transcription factor to guidance receptor that parallels Zic2 and EphB1 for the ipsilateral projection has not been identified. Although recent efforts have revealed candidate genes that directly regulate retinal expression of Plexin-A1 and NrCAM (T. Kuwajima & C. Mason, unpublished), whether they can indeed specify laterality in RGCs residing outside of the VT retina is not known. Alternatively, midline crossing may be an inherent property of RGCs, given that the contralateral projection appears to be the “ground state” in lower vertebrates, and may be difficult to eliminate when guidance or transcription factors are mutated.
**Genes that define ipsilateral and contralateral RGC identity**

While transcription factors can direct axon trajectory through regulation of guidance factor expression, they can also indirectly participate in these decisions by specifying cell identity. Although Zic2 has been shown to be both necessary and sufficient for inducing RGC axons to avoid the optic chiasm midline, a recent study demonstrated that Zic2 also plays a role in axon refinement at RGC targets via the serotonin transporter (SERT) (Garcia-Frigola and Herrera, 2010). In the retina, SERT expression is restricted to ipsilaterally projecting RGCs of the VT and dependent on direct transcriptional activation by Zic2. Through regulating serotonin availability, SERT has been shown to participate in axonal arbor remodeling, which is important in the segregation of ipsilateral and contralateral fibers projecting to dLGN and SC (Gaspar et al., 2003). As Zic2 has now been shown to control multiple stages of ipsilateral RGC growth from axon pathfinding (embryonic) to axon refinement (postnatal), it is likely to be a more upstream regulator of ipsilateral RGC identity.

**Patterning the ipsilateral and contralateral retinal domains**

Given the distinct identities of RGCs that project ipsilaterally versus contralaterally, the sectors in which they reside must be patterned to have similarly distinct properties. In vertebrates, the designation of nasal versus temporal retina is reflected in the complementary expression of winged helix transcription factors Foxg1 (BF-1) and Foxd1 (BF-2), respectively (Hatini et al., 1994). Studies in humans point to signals from outside the eye in establishing the nasotemporal axis (Lambot et al., 2005), while studies on early zebrafish eye development pinpointed FGF as this signal that, in turn, regulates Foxg1 expression (Picker et al., 2009). In mice, Foxd1 is broadly expressed in the temporal retina when the first ganglion cells are born.
(E11) and acts upstream of Zic2 and EphB1 in the genetic program associated with the uncrossed RGC projection (Herrera et al., 2004). Foxd1 has been further shown to imprint temporal identity within the mouse retina by directing the expression of EphA6 and ephrinA5, which play a role in rostrocaudal topographic mapping within RGC targets (Carreres et al., 2011).

Conversely, mice lacking Foxg1 have an increased ipsilateral projection (Pratt et al., 2004, Tian et al., 2008), suggesting a role for Foxg1 in repressing the ipsilateral and promoting the contralateral guidance pathways. Foxd1 is expressed in a region broader than the ipsilateral RGC domain, and both Fox genes are expressed in a gradient rather than in clearly delineated sectors. Thus, Foxd1 and Foxg1 could act as patterning genes, establishing general ipsilateral and contralateral domains that are further refined by downstream transcription factors such as Zic2. However, it is unclear whether the Fox genes indeed hold upstream positions within the transcription factor networks that specify ipsilateral and contralateral RGC identity or whether they control fate specification through gating proliferation and differentiation as they do in the cortex (Hanashima et al., 2002). Moreover, how Foxd1 and Foxg1 interact to establish the crossed and uncrossed domains of the retina remains to be determined. Both Foxd1 and Foxg1, as well as Zic2, are additionally expressed in the ventral diencephalon (Marcus et al., 1999, Herrera et al., 2004) and help to pattern the resident cell ensembles in and around the optic chiasm. Thus, any loss-of-function studies involving global knockouts that produce apparent disruptions in retinal decussation cannot distinguish between the roles these genes play in patterning the retina versus the optic chiasm.
A genetic network for regulating retinal decussation and RGC subtype identity

Despite our growing understanding of the genetic network that specifies the ipsilateral and contralateral RGC projections, many gaps remain where unidentified molecules are likely to play key functions (Fig. 1.6). In overexpression studies, Zic2 is more potent than EphB1 in converting laterality (Petros et al., 2009c), suggesting that Zic2 accomplishes this through regulating additional downstream factors important for midline guidance. Moreover, knockout mouse models for retinal guidance receptors all show only partial diverting of RGC projections to the opposite side. The transcriptional control of the contralateral projection and the patterning of the two domains giving rise to the ipsilateral and contralateral retina remains unclear. Finally, transcriptional regulators and adhesion molecules that mediate eye-specific fasciculation in the optic tract to targets and innervation of target regions, and that might also be responsive to neural activity, are not known. Thus, further identification of transcriptional regulators, guidance factors, and other genes important for ipsilateral and contralateral RGC subtype identity is needed.

Spatiotemporal differences in retinal ganglion cell production: the albino as a model

The albino mouse is a naturally occurring mutant that has been informative in studies of binocular visual development. Albinos have a hypopigmentation disorder that can result from different genetic mutations (King et al., 2003, Oetting et al., 2003). However, the universal phenotype of all forms of albinism is a decreased ipsilateral projection, resulting in perturbed binocular vision (Kaas, 2005). Additionally, anterograde labeling in Tyr\(^{-2J/c-2J}\) mice, which harbor a mutation in a key enzyme in melanin synthesis, tyrosinase, revealed that similar to the EphB1\(^{-/-}\) dLGN, a segregated patch forms within the albino dLGN; however, the position of this
patch differs, in that it is adjacent to the target zone of the late-born contralateral VT RGCs (Rebsam et al., 2012). Interestingly, the abnormal patches in both \( \text{Tyr}^{c-2J/c-2J} \) and \( \text{EphB1}^{-/-} \) mice derive from RGCs in the VT retina, and their segregation is dependent on normal retinal activity.

The picture emerging from the perturbations along the ipsilateral RGC projection pathway in the albino suggests a broader defect in ipsilateral RGC specification rather than a defect in midline axon guidance as seen in \( \text{EphB1}^{-/-} \) mice (Rebsam et al., 2009, Rebsam et al., 2012). Indeed, the number of VT cells that express the ipsilateral markers Zic2 and EphB1 is reduced in albino mice, in accordance with the reduction of the ipsilateral projection (Herrera et al., 2003a, Rebsam et al., 2012). Moreover, RGC neurogenesis is disrupted in the albino retina with respect to the timing of cell differentiation and regulation of cell cycle parameters (Webster and Rowe, 1991, Rachel et al., 2002, Tibber et al., 2006). Recent results from our lab suggest that a change in kinetics of RGC production occurs specifically in the VT retinal domain in \( \text{Tyr}^{c-2J/c-2J} \) embryos (Bhansali et al., submitted). A delay in the window of RGC production leads to fewer cells being born when VT RGCs are specified to project ipsilaterally and more being born at later ages when they project contralaterally.

The defects manifested in albinism are due to perturbed biogenesis or packaging of melanin in the retinal pigment epithelium (RPE), the monolayer of epithelial cells surrounding the neural retina. How these RPE defects alter retinal axon decussation at the optic chiasm has haunted the field for decades. One hypothesis is that factors in the melanogenic pathway in the developing RPE influence precursors in the neural retina to express genes that specify RGC laterality. As tyrosinase also mediates the production of L-DOPA, an intermediate product during melanin synthesis, L-DOPA has been proposed as such a key factor (Kralj-Hans et al., 2006, Lopez et al., 2008). Unanswered questions include how regulatory factors from RPE are
transferred to neural retina and whether developmental processes influenced by these factors include both neurogenesis and cell type specification. However, growing evidence suggests that both cell-extrinsic signaling and cell cycle regulation contribute to the defects in RGC subtype production seen in the albino mouse model.

**Mechanisms for neuronal subtype diversification**

Although the presence of multiple subtypes within a neuronal class adds another level of complexity to fate determination within the CNS, one can postulate that the same principles used in specifying classes of neurons are utilized in the specification of subtypes. The molecular identity of neuronal subtypes within the retina has not been explored until recently, and thus our understanding of retinal neuronal subtype specification is still lacking. Here, we can instead turn to other regions of the CNS, in which these questions have been explored in greater depth, to speculate on possible mechanisms at play within the retina.

**Transcriptional control of identity**

The evidence for transcription factor codes in the generation of neuronal subtype identity has blossomed in less than a decade. Within the spinal cord, motor neurons are divided into columns that innervate specific groups of muscles within the body axis. These columns can be further divided into motor neuron pools that innervate individual muscles and are defined by unique transcriptional profiles (Kanning et al., 2010). It is now known that specific combinatorial Hox protein profiles control the acquisition of both motor neuron columnar and pool identity (Dasen and Jessell, 2009).
In the cortex, subpopulations of projection neurons are also defined by layer-specific and sub-type specific transcriptional profiles (Molyneaux et al., 2007, Leone et al., 2008). These genes were mostly identified through large-scale in situ hybridization studies and gene expression analysis of microdissected regions of neocortex or purified neuronal subtypes (Molyneaux et al., 2007). Many of these genes, including Cux1 and 2, Lhx2, Brn2, ROR-beta, Opn3, and Foxp2 are expressed in specific layers; however, others are expressed in specific subtypes, such as Ctip2 in subcerebral neurons of layer V (Arlotta et al., 2005, Molyneaux et al., 2007). Whether these layer or subtype-specific genes play a role in fate determination of the cells in which they are expressed is still an open question. Some, such as Brn1 and Brn2 are involved in the differentiation of neurons within layer II-IV neurons, and double-knockout animals for these genes have decreased neuronal numbers within these layers as well as migration defects in the neurons that are produced (McEvilly et al., 2002, Sugitani et al., 2002).

An example of a transcription factor that appears as a true specifier of a specific neuronal subtype is Fezf2. Fezf2 is expressed in all subcerebral projection neurons from early stages of development through adulthood (Inoue et al., 2004, Arlotta et al., 2005). Fezf2−/− mice show complete absence of subcerebral projections neurons. Fascinatingly, layer V subcerebral neurons are not merely lost but instead appear to differentiate in layer VI neurons. The function of Fezf2 as a specifier of subcerebral projection neuron fate was further cemented by gain-of-function experiments using in utero electropration (Molyneaux et al., 2005). Premature expression of Fezf2 induces Ctip2 expression and migration failure of neuroblasts normally destined to become layer IV neurons, leading to their premature differentiation into layer V neurons.
Thus far, the only transcription in a subpopulation of RGCs that resembles the behavior of Fezf2 in subcerebral neuron specification is Zic2 as shown by the previously described gain- and loss-of-function experiments (Herrera et al., 2003a, García-Frigola et al., 2008, Lee et al., 2008). Zic2 promotes ipsilateral RGC identity through upregulating downstream effectors EphB1 and SERT, and overexpression of Zic2 appears to change the laterality of RGCs that are normally destined to project contralaterally. It has not been shown, however, whether Zic2 expression is simply required for conferring an ipsilaterality pathway from retina to LGN or if Zic2 truly specifies ipsilateral RGC fate, in which case, Zic2 overexpression should lead to the expression of all genes associated with ipsilateral RGC identity and also repress the expression of genes associated with contralateral identity.

**Heterogeneous progenitor pools**

As described in the earlier discussion of retinal development, multiple studies have pointed to the molecular heterogeneity of progenitor cells in multiple neural tissues. In addition to this mosaic heterogeneity found within a particular region, different types of neurons in the cortex and cerebellum are also known to arise from distinct progenitor zones, with some neurons migrating great distances laterally to reach their destination. Cortical neurons, for example, are divided into two broad classes: 1) glutaminergic projection neurons, which carry information to distant targets in subcortical, subcerebral, or other cortical regions; 2) GABAergic interneurons, which make local connections. While projections neurons are produced within the neocortical germinal zone of the dorsolateral wall of the telencephalon, interneurons originate from the distant ventral telecephalon and cortical helm (Anderson et al., 2002). Similarly, the morphologically and physiologically diverse cerebellar neurons have distinct origins.
GABAergic Purkinje cells originate from cerebellar ventricular zone progenitors, similar to cortical projection neurons (Mizuhara et al., 2010). In contrast, cerebellar granule cell precursors are generated within the embryonic rhombic lip and then migrate laterally into the external granule cell layer where they divide locally to give rise to postmitotic granule cells that migrate radially to their final positions within the internal granule cell layers (Wingate, 2001).

In the mammalian retina, neurons are thought to originate radially from progenitors located within the proliferative ventricular zone, which becomes juxtaposed to the RPE upon optic cup formation. Progenitor heterogeneity instead is regulated by spatiotemporal patterning cues that instruct the onset of proliferation and neurogenesis and molecular identity of individual progenitors. However, the presence of multiple proliferative zones has been described in lower vertebrates. In fish and amphibians, the ciliary margin zone (CMZ), a circumferential zone of cells located at the peripheral margin of the retina, is a continuous source of neurogenesis during postnatal growth as well as in response to injury (Kubota et al., 2002). However, the presence of the CMZ becomes progressively diminishes in vertebrate evolution, such that chicks have less CMZ cells than fish and amphibians and there is, at present, no evidence of a CMZ within mice.

The developing ciliary body, however, is a distinct compartment from the rest of the retina characterized by a unique gene expression profile, even in chick and mice (Trimarchi et al., 2009). Of particular interest, the ventral CMZ proliferates during metamorphosis in Xenopus and is the source of the ipsilaterally projecting RGCs that arise during this developmental stage (Beach and Jacobson, 1979, Marsh-Armstrong et al., 1999). Whether this compartment is a potential source of ipsilaterally projecting RGCs in mice or plays a role in their specification will be discussed further in Chapter 4.
Cell cycle regulation: G1 cyclins

As the regulation of cell proliferation and the timing of cell cycle exit can greatly influence the balance of cell types produced at specific developmental time points, factors that influence a cell’s decision to divide have been implicated in fate determination. However, studies within the last decade have illustrated that cell cycle regulators themselves can influence cell fate. The G1 phase of cell cycle is a particular focal point for environmental influences as it is during this phase that a cell makes the decision to divide (Sherr, 1995). In mammals, three D-type cyclins regulate the mid-G1 checkpoint. Interestingly, the expression of Cyclin D1, D2, and D3 during development is both overlapping and distinct (Ross, 2011). Moreover, null mutations for specific D-cyclins lead to entirely different developmental defects, suggesting a tissue and even cell-type specific dependence on individual cyclins. During cortical and cerebellar development, cyclin D1 and D2 have been shown to regulate the production of specific neuronal subtypes through regulation of cell cycle exit (Huard et al., 1999, Glickstein et al., 2007b, Glickstein et al., 2009). In the spinal cord, cyclin D1 continues to be expressed during the initial phase of post-mitotic motor neuron differentiation and plays a cell-cycle-independent role in promoting neuronal differentiation (Lukaszewicz and Anderson, 2011). The demonstration of in vivo transcriptional activity by cyclin D1 has opened even more potential functions of these genes during development (Bienvenu et al., 2010).

Prospectus of the thesis

Our understanding of the mechanisms regulating neuronal diversification within the retina has come a long way since the descriptive neuroanatomical studies of the late nineteenth century. In the investigation of how the binocular visual pathways develop, two very different
fields of study—the role of axon guidance in the development of the retinofugal projections and
the mechanisms underlying retinal histogenesis and neurogenesis—have converged upon the
question of how retinal neuronal subtypes giving rise to divergent axonal projections are
specified. Although we have now uncovered multiple signals that interact to control retinal axon
divergence at the optic chiasm and even some of the transcriptional regulators control the
expression of these signals, our understanding of RGC subtype specification with respect to
axonal projection is still in a primitive state.

This question cannot be easily addressed, however, before we acquire a better
understanding of how ipsilateral and contralateral RGCs are molecularly distinct. Given the
availability of powerful genomic techniques today, I chose to use a gene profiling approach
rather than a traditional candidate-based approach to characterize the molecular signatures of
ipsilateral versus contralateral RGCs. In Chapter 2, I will present a method I developed for
isolating these two RGC subpopulations based on their anatomical projections. Through gene
profiling of RGCs purified using this method, I have identified a number of genes that are
differentially expressed in ipsilateral versus contralateral RGCs, presented in Chapter 3. These
results demonstrated how these two RGC cell populations, conventionally thought to share a
common origin, project to the same targets, and differ only in their laterality, are characterized
by surprisingly different molecular signatures. Some of these genes that distinguish ipsilateral
and contralateral RGCs have relevance for growth and guidance, yet others reflect potential
differences in their origin and path to differentiation. In Chapter 4, I will finally return to the
question of ipsilateral versus contralateral RGC fate specification and present my results on the
role of one of these early genes, cyclin D2, in the production of ipsilateral RGCs.
FIGURES
Figure 1.1. Cellular diversity within the retina.

A) Drawing by nineteenth century neuroanatomist Santiago Ramón y Cajal depicting the structural organization of the retina and cellular morphologies within. B) Diagram depicting the six neuronal classes of the retina, organized in three cellular layers: outer nuclear layer (rod and cone photoreceptors), inner nuclear layer (amacrine, bipolar, and horizontal cells), ganglion cell layer (retinal ganglion cells) (Copyright © 2007 Pearson Education, Inc., Benjamin Cummings). C) Retinal ganglion cell subtypes have diverse morphologies that are associated with distinct molecular signatures, here defined by combinatorial expression of transcription factors Brn3a, Brn3b, and Brn3c (Badea and Nathans, 2011).
Figure 1.1. Cellular diversity within the retina.
Figure 1.2. Early eye morphogenesis in vertebrates.

A) A combination of extracellular signals from the extraocular mesenchyme and lens ectoderm pattern the optic vesicle into two domains, of which the distal/ventral domain develops into the future neural retina the dorsal into the retinal pigment epithelium (RPE). B) The surface ectoderm thickens and invaginates to become the lens pit whereas the optic vesicle folds inwards to form a two-layered cup, with domain-specific expression of the homeobox protein Vsx2 and the bHLH transcription factor Mitf within the developing neural retina and RPE, respectively (Fuhrmann, 2010).
Figure 1.2. Early eye morphogenesis in vertebrates.
Figure 1.3. Temporal competence transitions in retinal progenitor cells.

Retinal progenitor cells are thought to move through a series of intrinsically determined competence states during which the seven different cells classes are generated, here depicted for mouse development. E, embryonic day; P, postnatal day (Marquardt and Gruss, 2002).
Figure 1.3. Temporal competence transitions in retinal progenitor cells.
Figure 1.4. Retinal axon decussation at the optic chiasm of different species.

The topographic origin and relative proportion of crossed and uncrossed retinal projections varies across species (Mason, Kuwajima, and Wang, 2014).
Figure 1.4. Retinal axon decussation at the optic chiasm of different species.
**Figure 1.5.** Three phases of retinal ganglion cell axon outgrowth during mouse optic chiasm formation.

Horizontal view of the retinal axon projection through the optic chiasm. Chiasmatic radial glia form a palisade on either side of the midline and express glial markers such as RC2 and BLBP. The neurons expressing CD44 and SSEA-1 are positioned caudal to the chiasm and form a raphe rostrally through the midline.  

A) During the early phase (E12–13), the first RGCs from dorsocentral retina project both contralaterally and ipsilaterally. Crossed axons traverse the chiasm midline while transient uncrossed axons do not reach the chiasm palisade and turn laterally.  

B) During the peak phase (E14–17), the permanent ipsilateral projection arises from RGCs in ventrotemporal (VT) retina and the contralateral projection from RGCs in extra-VT retina. Both the ipsilateral and contralateral RGC axons approach the midline, where they then diverge.  

C) During the late phase (E18–P0), RGCs in VT retina project contralaterally, as do RGCs in extra-VT retina.  

E, embryonic day (Mason, Kuwajima, and Wang, 2014).
Figure 1.5. Three phases of retinal ganglion cell axon outgrowth during mouse optic chiasm formation.
Figure 1.6. Genetic network regulating decussation of retinal ganglion cell axons.

Genetic pathways within the ipsilateral (gray) and contralateral (white) retinal ganglion cell domains, including hypothesized interactions (dotted arrows). D, dorsal; V, ventral; N, nasal; T, temporal (Mason, Kuwajima, and Wang, 2014).
Figure 1.6. Genetic network regulating decussation of retinal ganglion cell axons.
Chapter 2. Purification of embryonic ipsilateral and contralateral retinal ganglion cells

Introduction

Previous work conducted by members of the Mason Lab led to the discovery of two different guidance programs that directed the growth of the ipsilateral and contralateral projections at the mouse optic chiasm: the EphB1 and EphrinB2 interactions that repel ipsilateral axons from the midline and the NrCAM/PlexinA1 complex that reverses the inhibitory Sema6D signal to promote contralateral axon growth through the midline (Williams et al., 2003, Williams et al., 2006b, Kuwajima et al., 2012). Our lab and others continued to look for new genes involved in directing midline crossing as well as proper growth at multiple points along the retinogeniculate pathway. However, all of these studies utilized a candidate-based approach, focusing on genes with a predicted contribution to axon guidance based on their previously characterized role in other decussating pathways in the nervous system, such as the ventral nerve cord of Drosophila, or in similar pathways of other species, such as the Ephrin-B and EphB-mediated repulsion at the Xenopus optic chiasm (Nakagawa et al., 2000). While many of these genes are indeed expressed in the developing mammalian binocular pathway, their functions are not always preserved. For example, the robo-slits interactions that are critical to midline repulsion of ipsilateral axons and post-crossing commissural axons in Drosophila are not important for midline repulsion at the optic chiasm (Dickson and Gilestro, 2006). Instead, these molecular interactions create a repulsive corridor in the mammalian optic chiasm region that
promotes fasciculation and guides axons as they approach the midline (Erskine et al., 2000, Plump et al., 2002, Plachez et al., 2008).

To continue our efforts at delineating the molecular players that direct retinal axon growth through the chiasm and to their appropriate target zones within the dLGN, our lab has pursued an ongoing interest in conducting an unbiased screen of ipsilateral and contralateral RGCs to identify new candidates that have not been previously described in other systems. A challenge to such studies is that ipsilateral RGCs constitute a very small population of cells within the retina (only ~3-5% of the final RGC number and ~10% at E16.5). Thus, the ipsilateral RGC population is particularly sensitive to contamination by other cell types when using anatomical isolation approaches. In an earlier attempt to tackle this question, former postdoctoral fellow Alexandra Rebsam executed a microarray screen of cells residing in the peripheral quadrants of E15.5 mouse retina (unpublished). Using our standard dissection method for preparing explant cultures, she dissected dorsotemporal (DT), ventrotemporal (VT), dorsonasal (DN), and ventronasal (VN) quadrants of neural retina and extracted RNA from the quadrants. However, the subsequent microarray analysis of these samples yielded very few genes that were enriched in the VT quadrant and failed to detect the important ipsilateral marker Zic2. We deduced that the lack of sensitivity of this approach was due to the heterogeneous population of cells present in the retina at this age. Specifically, these cells include RGCs and retinal progenitors, which predominate, as well as horizontal cells and other cell types that have also begun to differentiate (Cepko, 1999). Thus, gene expression differences between ipsilateral and contralateral RGCs may have been diluted by the gene expression patterns of other cell populations. Even using an approach such as laser capture microdissection of fresh retinal sections may not completely circumvent this problem; as early as E14.5, ipsilateral RGCs within
the VT periphery are already intermingled with some RGCs that project contralaterally and thus would be difficult to cull out.

My main interest upon joining the lab was to investigate how ipsilateral and contralateral RGCs are differentially specified and what genes operate upstream of the guidance programs already known to regulate retinal axon decussation. Thus, conducting a screen of these two RGC subpopulations would provide me with a list of candidates that may regulate fate determination, as well as later events in axon guidance. As Alexandra Rebsam had already established a successful method for gene chip analysis in her studies, my focus was on developing a cleaner method for isolating ipsilateral versus contralateral RGCs. To this end, I explored both genetic and anatomical approaches to specifically label ipsilateral and contralateral RGCs. In this chapter, I will present a new method for purifying RGCs based on their axonal projections through a combination of retrograde labeling and fluorescence-activated cell sorting (FACS).

**Results**

*Zic2-EGFP BAC transgenic mouse does not specifically label ipsilaterally projecting RGCs*

The first and simplest approach I considered for purifying ipsilateral and contralateral RGCs was to use a reporter mouse in which each population is labeled using a genetic marker. This approach, in conjunction with FACS, has been used successfully in retina and other CNS regions to isolate cells expressing a particular reporter gene (Marsh et al., 2008, Pastrana et al., 2009, Kay et al., 2011). While NrCAM and PlexinA1 have specific roles for promoting midline crossing of RGC axons, they are expressed in both contralateral and ipsilateral RGCs and thus are not good markers for contralateral RGCs. Isl2 appears to be a specific marker of
contralateral RGCs, however, its expression is extremely dynamic and only ~33% of contralateral RGCs express Isl2 at any given point in time (Pak et al., 2004). Thus, isolation techniques focusing on Isl2 would harvest only a subset of contralateral RGCs. Brn3a appears to be expressed only in RGCs projecting contralaterally and as such, seems to be the most specific and inclusive contralateral RGC marker (Quina et al., 2005). Thus far, three genes are predominantly expressed in ipsilateral RGCs from E14-16: the guidance receptor EphB1 (Williams et al., 2003), transcription factor Zic2 (Herrera et al., 2003a), and the serotonin transporter SERT (or Slc6a4) (Gaspar et al., 2003, García-Frigola and Herrera, 2010).

At the start of this study, I decided to use Zic2 as the marker for purifying ipsilateral RGCs due to the availability of the Tg(Zic2-EGFP)HT146Gsat mouse through GENSAT (Gong et al., 2003). This transgenic mouse carries a bacterial artificial chromosome (BAC) in which coding sequences for EGFP have replaced the Zic2 coding sequence but the cis regulatory elements of the endogenous gene are preserved. To assess the specificity of GFP reporter expression against endogenous Zic2 protein expression, I immunostained retinal sections of Zic2-EGFP embryos with antibodies against Zic2 and Isl1/2 (differentiated RGC marker) at E15.5, the peak of Zic2 expression within ipsilateral RGCs. However, my results showed that the GFP reporter signal does not faithfully recapitulate endogenous Zic2 protein expression (Fig. 2.1). While the developing ciliary body, which typically expresses lower levels of endogenous Zic2 protein, is strongly positive for the reporter signal (Fig. 2.1a, bracket), GFP expression is absent in the majority of Zic2⁺Isl1/2⁺ RGCs and present only in the most peripheral RGCs that express Zic2 protein (Fig. 2.1a, arrows). Moreover, immunostaining against GFP to detect lower levels of reporter expression revealed that RGCs and axons from not only ventral but also dorsal retina are positive for GFP signal (Fig. 2.1b), even though dorsal RGCs do not express Zic2.
Thus, GFP expression within the Zic2-EGFP BAC is not specific to and does not encompass the full span of Zic2-expressing RGCs that project ipsilaterally within the retina. Therefore, this reporter mouse did not prove to be useful for isolating ipsilateral RGCs by FACS.

*Rapid retrograde labeling of RGCs from the optic tract*

As an alternative to a genetic approach for labeling ipsilateral and contralateral RGCs, I next explored the possibility of applying neuroanatomical techniques previously used for tracing axonal projections to the isolation of these cell populations. To visualize the positions and numbers of ipsilateral and contralateral RGCs within the retina, our lab has utilized a retrograde labeling method in which axons from one optic tract are labeled with a fluorescent dye, which is subsequently transported to cell bodies within the ipsilateral or contralateral retina. In this procedure, the optic tract is accessed by removal of the palate and exposure of the ventral brain, in an isolated head preparation (Fig. 2.2b; see *Ch. 6, Materials and Methods*). Our established method for retrograde labeling of unfixed RGC axons made use of rhodamine-conjugated dextran of 6000 molecular weight (RD6000) and required a 2-4 hour incubation at room temperature (RT) followed by overnight incubation at 4°C for complete labeling (Rachel et al., 2002). However, prior to the start of my project, newer batches of the custom-made RD6000 (Molecular Probes) no longer worked in retrograde labeling experiments. Moreover, despite the use of oxygenated artificial cerebral spinal fluid (ACSF) to promote cell survival, the required overnight incubation for complete labeling is not conducive to maintaining tissue integrity for subsequent gene profiling experiments. Thus, I set out to develop a retrograde labeling protocol optimized for both specificity of labeling and cell survival, as needed for FACS and subsequent gene expression analysis.
To establish a new retrograde labeling protocol, I tested several commonly used tracers for axon labeling within unfixed tissue using our established retrograde labeling protocol with overnight incubation: RD6000, rhodamine-conjugated dextran molecular weight 3000 (RD3000), fluorescein-conjugated dextran molecular weight 3000 (FD3000), and cholera-toxin-subunit B (CTB) conjugated to AlexaFluor 488 (data not shown). While RD6000 and CTB-Alexa488 failed to label RGC cell bodies, FD3000 specifically labeled RGC cell bodies within the VT peripheral crescent of the ipsilateral retina and throughout the contralateral retina with lower density in the VT, as expected. Interestingly, RD3000 labeled tissue showed diffuse and non-specific labeling of cells throughout both retinas, suggesting that the dye leaked out of labeled RGCs during incubation.

As FD3000 specifically labeled RGC cell bodies, it served as an exciting candidate for isolating ipsilateral and contralateral RGCs. However, our established protocol uses an overnight incubation that is incompatible with cell survival and RNA expression studies. I next attempted to ascertain whether it would be possible to label RGC bodies with shorter incubation periods. While RGC cell bodies were fully labeled with overnight incubation, only partial labeling of axons was accomplished with shorter incubation times at RT alone (between 1 and 6 hours). Despite its promising use for labeling axonal trajectories for anatomical studies, FD3000 is not a viable dye for use in isolating specific RGC populations.

I next posited that if overnight incubations with RD3000 resulted in diffuse non-specific staining due to leakage, shorter incubations might achieve more specific labeling. In addition to the traditional incubation protocol, I tested a series of incubation times from 1hr to 6 hours (with 15 minute increments) at RT, without overnight incubation. In contrast to the diffuse non-specific labeling seen with overnight incubations, short incubations of 1.5-4 hours resulted in
highly specific and strong labeling in RGCs (Fig. 2.2c). Cryosections of retrogradely labeled retinas from E16.5 embryos further confirmed that labeling was specific to the RGC layer and to cells within the VT region of ipsilateral retinas and extra-VT regions of contralateral retinas (Fig. 2.3).

Thus, my experiments established a protocol for rapid and specific retrograde labeling of RGC soma from E16.5 optic tract within 2 hrs of RD3000 dye application (Fig. 2.2a and b). These conditions are optimal for preserving tissue health for subsequent cell sorting experiments. I also demonstrated that FD3000 is a useful alternate dye for analysis of RGC projections, although the required overnight incubation renders it sub-optimal for cell sorting experiments.

*Isolation of retrogradely labeled RGCs from ipsilateral and contralateral retinas by cell sorting*

Using the above method to retrogradely label RGCs, I then dissected out retinas ipsilateral or contralateral to the labeled OT from two E16.5 litters (12-15 embryos total). The fluorescence signal within labeled retinas was examined and only retinal pairs with specific and sufficient RGC labeling were used for cell sorting (Fig. 2.4a). Pooled ipsilateral and contralateral retinas (8-10 each) were dissociated with papain to obtain a single cell suspension, and rhodamine-dextran positive cells were then purified by FACS. Two litters of retrogradely labeled embryos yielded approximately 3000 ipsilateral RGCs and 20,000 contralateral RGCs (Fig. 2.4b).

To assess the purity of FACS-purified RGCs, I plated the FACS-sorted cells on polyornithine-coated coverslips without laminin and cultured them for 1 hour to allow for
attachment but still minimize gene expression changes. Fixed cells were immunostained with an antibody against Isl1/2 to visualize differentiated RGCs. The vast majority of FACS sorted cells expressed Isl1/2, suggesting minimal contamination with other retinal cell types, such as retinal progenitor cells and glia (Fig. 2.5). Subsequent qPCR analysis showed that rhodamine-labeled RGCs from ipsilateral retinas express high levels of Zic2 and SERT as compared to those from contralateral retinas (see Chapter 3, Fig. 3.1a). Thus, rapid retrograde labeling from the OT followed by FACS is a clean and effective way to purify ipsilateral and contralateral RGCs during development.

Discussion

In this chapter, I have described a new method for isolating embryonic ipsilateral and contralateral RGCs based on the laterality of their axonal projections (Fig. 2.6). In postnatal animals, retrograde labeling can be performed by injecting axonal tracers into the dLGN or superior colliculus in vivo (Dräger and Olsen, 1980, Pak et al., 2004). However, organization of these targets is only rudimentary at embryonic ages, as most RGC axons have not reached the dLGN. Thus, retrograde labeling of RGCs cannot be performed in utero from the target, as described in the purification of cortical neuronal subtypes (Arlotta et al., 2005), and requires removal of embryos from the mother and exposure of the ventral surface of the brain to access the OT. The purification method I have developed utilizes a rapid retrograde labeling technique that allows for completion of cell purification within 7 hours, from removal of the embryo to RNA extraction or cell culturing, thereby allowing for optimal cell survival under ex vivo conditions.
I further demonstrate that this anatomical approach for purifying ipsilateral and contralateral RGCs leads to minimal contamination with non-RGC cell types, a problem that plagued our lab’s previous microarray attempt using retinal peripheral quadrants. Moreover, this approach allows for complete separation of ipsilateral and contralateral RGCs, which cannot be accomplished by dissection or laser capture of VT peripheral retina, as there is inevitably some intermingling of contralateral RGCs and ipsilateral RGCs within the VT region even at early developmental time points.

While retrograde labeling from the OT can be performed at embryonic ages, a significant cohort of ipsilateral RGCs cannot be labeled earlier than E16. Thus, this approach is not as effective for isolating RGCs at earlier developmental time points. Moreover, retrograde labeling from the OT selects for RGCs whose axons have reached the OT and are thus more differentiated. To isolate newly born cells for identification of genes important for early stages of RGC development, alternative approaches are required, such as using reporter mouse lines for early genes that define ipsilateral or contralateral RGCs. Identification of additional ipsilateral and contralateral markers and generation of reporter lines for these genes would be needed before such experiments are possible.

At the beginning of my experiments, I examined the one transgenic reporter line available to me of the known ipsilateral and contralateral RGC markers: the Zic2-EGFP BAC mouse. However, subsequent analysis of GFP expression revealed that it does not sufficiently recapitulate endogenous Zic2 expression and is not specific to ipsilateral RGCs. The signal seen in dorsal RGC axons likely results from GFP perdurance in cells that express Zic2 prior to terminal differentiation, as lower levels of Zic2 expression is found in retinal progenitor cells throughout the retina, especially during early development. Moreover, GFP signal was not
detected in many Zic2+ RGCs, suggesting that the BAC gene is not regulated in the same manner as the endogenous locus. For my gene profiling experiments, I chose to utilize retrograde labeling at E16.5 in hopes that RGCs that have crossed the chiasm still have detectable expression of genes turned on earlier in differentiation.

In the following chapter, I will demonstrate one use of the cell isolation method I have developed: gene expression profiling of ipsilateral and contralateral RGCs (Fig. 2.6g). However, these approaches can be also used for other experiments requiring purified sub-populations of RGCs, such as experiments to determine the contribution of cell-extrinsic effects of contralateral RGCs on ipsilateral RGC gene expression or vice-versa in co-culture conditions (Fig 2.6h). Similarly, purified ipsilateral or contralateral RGCs can also be used for in utero transplantation experiments in mutant genetic backgrounds to determine whether genes required for their proper projection operate cell-autonomously (Fig. 2.6i). As a proof of principle, it would be necessary to determine whether ipsilateral and contralateral RGCs purified by this approach grow well in culture and whether they respond to normal axon guidance cues, such as Ephrin-B2 (Petros et al., 2009b), demonstrating the preservation of their normal functional behaviors.
FIGURES
Figure 2.1. Zic2-EGFP BAC transgenic mouse does not specifically label ipsilaterally projecting retinal ganglion cells.

A) Coronal cryosections of E15.5 VT retina of Zic2-EGFP mouse, immunostained with antibodies against Zic2 (ipsilateral RGCs), and Isl1/2 (all RGCs). GFP reporter signal is present in the ciliary body (red bracket) but only some Zic2⁺ RGCs (red arrows). B) Coronal cryosections of E15.5 retina of Zic2-EGFP mouse, immunostained with antibodies against GFP shows GFP expression in dorsal (white arrow) in addition to ventral RGC axons.
Figure 2.1. Zic2-EGFP BAC transgenic mouse does not specifically label ipsilaterally projecting retinal ganglion cells.
Figure 2.2. Retrograde labeling of retinal ganglion cells at E16.5 with RD3000.

A) Flowchart listing steps for retrograde labeling of RGCs from the optic tract with rhodamine dextran 3000 MW dye (RD3000). B) Diagram depicting ventral view of embryonic brain and position of RD3000 placement on cut unilateral optic tract. C) Whole-mount preparation of E16.5 retinas. RD3000 fully labels axons and cell bodies of E16.5 RGCs within 2 hours of incubation time from dye application to the optic tract. Extra-VT RGCs are labeled in retina contralateral to labeled optic tract and VT RGCs in ipsilateral retina.
Figure 2.2. Retrograde labeling of retinal ganglion cells at E16.5 with RD3000.
Figure 2.3. Rhodamine-dextran 3000 specifically labels retinal ganglion cells.

Coronal cryosections of E16.5 retinas retrogradely labeled with RD3000 show specific labeling of contralateral (A, C) and ipsilateral RGCs (B, D) in the appropriate retinal domain.
Figure 2.3. Rhodamine-dextran 3000 specifically labels retinal ganglion cells.
Figure 2.4. FACS purification of retrogradely labeled ipsilateral and contralateral RGCs.

A) Fresh E16.5 retinas labeled with RD3000 are screened for appropriate labeling of ipsilateral and contralateral RGCs prior to FACs. B) FACS purification ipsilateral and contralateral RGC populations retrogradely labeled with RD3000 with DAPI exclusion. ~3000 ipsilateral and ~20,000 contralateral RGCs (P4 gate) are purified from 2 litters of E16.5 embryos.
**Figure 2.4.** FACS purification of retrogradely labeled ipsilateral and contralateral RGCs.
Figure 2.5. FACS purified cells retrogradely labeled with RD3000 express RGC markers.

Acute staining shows RGC marker (Isl1/2) expression in FAC purified cells from retrogradely labeled retinas.
Figure 2.5. FACS purified cells retrogradely labeled with RD3000 express RGC markers.
Figure 2.6. Summary of ipsilateral and contralateral RGC purification method.

Schematic of retrograde labeling method (A-C), cell purification (D-F), and potential applications for purified cell populations (G-I).
Figure 2.6. Summary of ipsilateral and contralateral RGC purification method.
Chapter 3. Molecular identity of developing ipsilateral and contralateral RGCs

Introduction

Our present understanding of the unique identities of ipsilateral and contralateral RGCs centers around the laterality of their axonal projections and the respective guidance programs that set up these projections. As discussed in the previous two chapters, despite our growing understanding of the genetic network that specifies the ipsilateral and contralateral RGC projections, many gaps remain within this network where unidentified molecules are likely to play key functions. Of the known retinal guidance receptors, knockout mouse models only show partial changes in laterality (Williams et al., 2003, Williams et al., 2006a, Erskine et al., 2011, Kuwajima et al., 2012). Moreover, the molecular interactions between transcription factors (e.g., Zic2 and Isl2), downstream effectors (e.g., EphB1, Neuropilin, NrCAM, and PlexinA1) and upstream patterning genes (e.g., Foxd1 and Foxg1) within this genetic network have proven difficult to identify, suggesting that unknown intermediate genes bridge these gaps. For example, in overexpression studies, Zic2 is more potent than EphB1 in switching RGC projection laterality (Petros et al., 2009b) and thus may regulate additional downstream factors in the uncrossed guidance program.

Although a number of different axon guidance genes have been shown to be important for midline crossing of contralateral RGC axons, these genes are also expressed in ipsilateral RGCs. The only known specific markers of contralateral RGCs are the LIM homeodomain
transcription factor Isl2 and the Pou-domain transcription factor Brn3a (Pou4f1). However, loss of Brn3a does not influence RGC axon laterality (Quina et al., 2005), and while Isl2 is important for contralaterality in late-born VT RGCs, it is not required for the major contralateral projection from extra-VT retina (Pak et al., 2004). Thus, a transcription factor that specifies contralateral identity throughout the retina has not yet been identified. Preliminary studies by Takaaki Kuwajima, a postdoctoral fellow in the lab, suggest that the SoxC family of transcription factors (Sox 4, 11, 12) may direct the expression of NrCAM and PlexinA1 expression to promote axon growth through the inhibitory chiasm environment. However, it is unclear whether these genes play a role in controlling other axon guidance genes, directly determining contralateral RGC fate, or promoting non-specific RGC differentiation. Finally, transcriptional regulators and adhesion molecules that mediate fasciculation of eye-specific RGC axons in the optic tract and innervation of target regions are still unknown.

Thus, further identification of transcription factors, guidance factors, and other genes important for ipsilateral and contralateral RGC subtype identity is needed. Moreover, identification of additional genes that specifically mark ipsilateral and contralateral RGCs would greatly facilitate further studies of how these two RGC subtypes develop. One approach to tackling these questions is via a thorough analysis of the molecular signatures of ipsilateral and contralateral RGCs to identify genes that distinguish them. Such an approach has proven very useful in recent studies of other neuronal subtypes, such as cortical projection neurons (Arlotta et al., 2005) and amacrine cells (Kay et al., 2011), and has been particularly successful in uncovering transcriptional networks that regulate postmitotic cell fate acquisition.

In this chapter, I will present my efforts at combining the ipsilateral and contralateral RGC purification method I developed (Chapter 2) with gene profiling to identify the genes that
distinguish ipsilaterally and contralaterally projecting RGCs during the critical period of axon outgrowth and midline crossing in an attempt to fill in gaps for the genetic network regulating ipsilateral and contralateral RGC identity, axon guidance, and eye-specific innervation.

Results

Expression profiling of ipsilateral and contralateral RGCs confirms differential expression of known ipsilateral and contralateral markers and reveals novel differences

I conducted expression profiling of purified ipsilateral and contralateral RGC populations acquired using the retrograde labeling and cell sorting methods described in Chapter 2 (Fig. 2-6). Due to the small number of ipsilateral RGCs isolated with this method (~3000 FACS events for 2 litters of retrogradely labeled embryos), cDNA prepared from these samples was amplified prior to gene chip hybridization. To verify the purity of the samples, I measured expression levels of Zic2 and SERT by qRT-PCR (Fig. 3.1a). The three biological replicates (i.e., independent rounds of retrograde labeling and FACS) that showed the most robust enrichment in Zic2 and SERT expression in ipsilateral RGCs were selected for microarray analysis.

Gene ontology analysis of microarray results revealed that many differentially expressed genes are involved in developmental processes, including regulation of gene expression, cell proliferation, cell cycle progression, and cell differentiation (Table 3.1). As expected, most genes were expressed at similar levels in ipsilateral and contralateral RGC samples, including the pan-RGC transcription factors Brn3b and Isl1 (Pan et al., 2008). However, 339 genes were differentially expressed between the two populations by 2-fold or more (corrected p-value <0.05, Benjamini-Hochberg) (Fig. 3.1b) and included genes known to be enriched in ipsilateral RGCs.
such as Zic2 (5.78-fold) and SERT (4.49-fold), as well as contralateral RGC marker Brn3a (2.20-fold) (Table 3.2, bold entries). The other contralateral RGC marker, Isl2, was only elevated by 1.5-fold and did not make the 2-fold cutoff; however, this may because Isl2 is only expressed in only a subset of contralateral RGCs.

The microarray gene list also included genes previously not characterized with regards to their differential expression in RGC subpopulations, most of which were elevated in ipsilateral RGCs (Fig. 3.1c). In total, 299 genes (404 probe sets) were found to be enriched in ipsilateral RGCs and 40 genes (47 probe sets) in contralateral RGCs, using a 2-fold cutoff (Fig. 3.1b), suggesting that an extensive genetic program is activated to generate ipsilateral RGCs. An alternative explanation for these findings is that, because ipsilateral RGCs reside in a more spatially restricted domain within the retina, some of these genes may have region-specific expression patterns.

For initial microarray validation, I analyzed the expression levels of 11 new gene candidates (9 ipsilateral high and 2 contralateral high) in FACS-derived RGCs by quantitative RT-PCR. Of these 11 genes, 10 were confirmed to be enriched in the RGC subpopulation as seen by gene profiling, demonstrating inter-platform reproducibility of the gene expression detection by microarray (Table 3.2). In summary, gene profiling of purified ipsilateral and contralateral RGCs at E16.5 revealed that these two RGC subsets are defined by unique molecular signatures, providing an extensive list of candidate genes that may be differentially expressed in these two cell populations.
Selection of candidate genes for expression validation

To validate the expression of candidate genes in vivo, I used in situ hybridization (ISH) and immunohistochemistry (IHC), which provide information regarding the spatiotemporal pattern of expression as well as co-localization with known ipsilateral or contralateral RGC markers. As the RGCs that I isolated for gene profiling have already completed their midline choice, I expected to identify genes downstream of the main transcriptional regulators. However, upstream or parallel genes may be identified if there is still residual levels of expression in RGCs that have axonal projections reaching the optic tract.

The primary purpose of this microarray screen is to identify additional regulators of axon guidance at the midline and target, including guidance molecules and transcription factors, other genes that functionally distinguish ipsilateral and contralateral RGCs, and, finally, genes that specify ipsilateral and contralateral RGC fate. Thus, of the 339 differentially expressed genes, I focused on 5 groups: transcription factors involved in neuronal development (e.g. Sox2, Lhx2, Math5, Tbx20), signaling pathways prominent during development (e.g. Igf1, Igfbp5, Fgf12, Ptch1), axon guidance related genes (e.g. Sema3e, Sema4d, Sema5b, Sema7a), cell cycle regulators (e.g., Ccnd1, Ccnd2), and other cell surface or secreted molecules that may be related to cell differentiation (e.g., Gja1, Sparc, Zip6/Liv-1, Napb). Representative genes of these groups are shown in Table 3.3.

Within these functional groups, I further narrowed the list of candidates using two approaches: 1) a literature search to determine whether a candidate has a known role in axon guidance, cell differentiation, or cell fate specification (especially in neurons); 2) an expression atlas search (Allen Brain Atlas and GenePaint.org) to determine whether a candidate gene is
present in the retina. However, these provided only rough guidelines for culling candidates because of their correlated caveats: 1) candidate genes may play a novel role in RGC subtype development that has not been previously described in other systems; 2) expression atlases, while extensive, do not have information on all of the candidate genes I investigated and often did not include sections through the retina; moreover, most available images are of sagittal sections, which is the least informative plane of section for determining differential expression in ipsilateral and contralateral RGCs. Thus, I conducted further expression analysis on some genes that have strong potential of biological relevance in the retina even if they did not pass selection criteria described above.

Altogether, I analyzed the expression patterns of 32 genes that have not been previously reported as differentially expressed in ipsilateral and contralateral RGCs; some of these genes have never been characterized in developing retina (Tables 3.4-3.6). I obtained previously published riboprobe expression plasmids for Math5 (L. Gan, University of Rochester), Fzd5, Tbx20 (G. Papaioannou, Columbia University), Sema3e, and Sema5b (A. Kolodkin, Johns Hopkins University) from other labs. For the remaining genes, I designed and generated my own riboprobe expression plasmids, targeting the 3’ region of each gene’s cDNA if possible, as these sequences tend to be less conserved between related genes and would lead to less cross-reactivity (see Chapter 6, Materials and Methods). In the following sections, I will describe the observed expression patterns of the most interesting gene candidates.

**Genes selectively expressed in contralateral RGCs**

At present, very few specific markers of contralateral RGCs have been identified, and no gene has been shown to specify contralateral identity throughout the retina. Thus, I was keen to
identify genes that are specifically expressed in contralateral RGCs. To confirm that select
candidate genes are indeed specifically expressed in contralateral RGCs, I analyzed the mRNA
expression pattern of the gene of interest at E15.5 (peak of Zic2 expression) by ISH followed by
co-immunostaining with ipsilateral RGC marker Zic2 and pan-RGC marker Isl1 (using an
antibody that targets Isl1 and 2) within the same sections. Of the 7 new contralateral genes
tested (Tables 3.4 and 3.6 lower panel), the following genes showed exclusive expression in
contralateral RGCs (Isl1/2\(^+\)Zic2\(^-\) cells): transcription factor Tbx20, cell surface/secreted protein
Sema3e, and growth factor Igf1 (Fig. 3.3a-c). Fgf12 is also enriched in RGCs outside of the
Zic2\(^+\) zone and only shows weak expression in VT RGCs (Fig. 3.3b).

Interestingly, Fgf12 and Igf1 expression is weaker in the most peripheral RGCs of DT
retina, suggesting that the youngest DT RGCs (Fig. 3.3a and b, adjacent to dashed red line) have
not yet accumulated as much of these transcripts as the more central RGCs. Tbx20 and Sema3e
are even more centrally expressed and appear to have more mosaic expression. Coincidentally,
Tbx20 was recently identified by Andy Huberman’s group (UCSD) as a potential marker for a
functional RGC subset that only projects contralaterally (A. Huberman, personal
communication). Thus, Sema3e may similarly mark a functional subtype of contralateral RGCs
that is present only in central retina or arises later in development. Alternatively, Tbx20 and
Sema3e expression may be delayed in RGCs and thus only seen in the oldest RGCs present at
this age. Consistent with either of these explanations, both Sema3e and Tbx20 are not yet
expressed in RGCs at E13.5 (data not shown). Additional ISH analysis at earlier and later
developmental ages are needed to resolve the expression time course for these genes and to
determine whether their expression would expand as greater numbers of RGCs mature.
Together, Igf1, Fgf12, Tbx20, and Sema3e represent 4 new contralateral RGC markers during the peak period of axon outgrowth, when ipsilateral and contralateral RGCs diverge. None of these genes have been studied in the context of RGC development. Tbx20 has been studied extensively in heart development (Plageman and Yutzey, 2005) and cranial motor neuron migration (Song et al., 2006). The Igf and Fgf signaling pathways have broad mitogenic and cell survival effects during development, tissue repair, and tumor growth. Interestingly, unlike most secreted FGF family members, Fgf12 localizes to the nucleus (Smallwood et al., 1996), and thus may play a cell-autonomous role in RGCs. Finally, the expression and guidance roles of semaphorin family members have been demonstrated at multiple points along the path of retinal axons (Callander et al., 2007). Sema3e is known to be expressed at the optic chiasm, where it may influence midline crossing decisions. Here, I show that it is also expressed in a subset of contralateral RGCs, raising the question of why this repulsive guidance cue is also expressed by RGCs. One answer to this question is the recent demonstration that RGC-secreted Sema3e regulates retinal angiogenesis through interactions with Plexin-D1 expressed on endothelial cells of sprouting blood vessels (Callander et al., 2007). However, whether Sema3e has a further function in regulating contralateral RGC axon growth and guidance has not been explored. Thus, these microarray experiments have great potential for revealing novel genes important for contralateral RGC function.

Ipsilateral RGCs express early developmental markers

Although Zic2, EphB1, and SERT have been shown to be predominately expressed in ipsilateral RGCs from E14-17, their genetic relationship is still unclear. A large group of genes enriched in ipsilateral RGCs in the microarray were transcription factors known to be important
in the development of retinal progenitor cells or RGC precursors. These candidates include the pluripotency gene Sox2, stem cell marker Klf4, retinal precursor gene Math5 (Atoh7), eye field transcription factor (EFTF) Lhx2, Zic family members Zic1 and Zic3, amongst many others.

Similarly, ipsilateral RGCs were enriched in a number of cell cycle regulators, including cyclins and cyclin-dependent kinases (Table 3-3). These findings were simultaneously interesting and alarming, as they suggest two possibilities: 1) the ipsilateral RGC sample is disproportionately contaminated with RNA from proliferating cells, or 2) these genes are indeed enriched in ipsilateral RGCs.

As progenitor genes were a significant component of the ipsilateral RGC gene expression program, I was particularly interested in further analyzing the expression of these genes to determine whether there is indeed a contamination issue with my microarray or if these genes hint at a functional genetic program unique to ipsilateral RGCs. To validate the expression pattern of genes enriched in ipsilateral RGCs in the microarray, I again used ISH analysis on coronal sections of E15.5 retina, co-immunostaining for RGC markers. Here, I selected transcription factors that participate in CNS development as discussed above, as well as cell cycle regulators, developmental signaling molecules, family members of axon guidance molecules, and other secreted or membrane-expressed genes that may play a role in differentiated RGC function (Table 3.5, 3.6 upper panel, 3.7). Of the 23 new ipsilateral genes tested, many were expressed in proliferative zones within the retina, as expected. However, 3 genes showed additional expression in ipsilateral RGCs (Sox2, Math5, Igfbp5, Fig. 3.4, Table 3.5 upper panel) as well as 4 other genes at a weaker level or within a more limited developmental window (Fig. 3.5, Table 3.5 lower panel).
The most striking expression pattern of this group of validated genes is that of insulin-like growth factor binding protein 5 (Igfbp5). Igfbp5 is expressed in RGCs of the Zic2\(^+\) RGC zone throughout the retina (Fig. 3.4c). Closer examination reveals that Igfbp5 is most strongly expressed in Zic2\(^+\)Isl1/2\(^+\) RGCs of this region, but not all Zic2\(^+\) RGCs express Igfbp5, and some Zic2\(^-\) cells also express lower levels of Igfbp5 and are located at the junction between the RGC and neuroblastic layers (Fig. 3.4c, high power). Igfbp5 is also expressed in the most peripheral contralaterally projecting RGCs (Zic2\(^-\)Isl1/2\(^+\)) of DT retina, although these cells are much fewer than the VT RGCs that express Igfbp5. Interestingly, Igfbp5\(^+\) cells correspond to the most peripheral DT RGCs that lack Igf1 expression seen earlier (Fig. 3.3a). Thus, Igfbp5 and Igf1 have a complementary pattern of expression in E15.5 retina, suggesting that these two components of the Igf signaling pathway have complementary or antagonistic functions at this age.

Two other ipsilateral RGC-enriched microarray candidates that showed an interesting expression pattern are the transcription factors Sox2 and Math5. Sox2 is expressed in neuronal progenitors during CNS development, including the retina, but is downregulated during the final division as the progenitor becomes postmitotic (Pevny and Nicolis, 2010). Indeed, I observed homogeneous Sox2 expression within the proliferative regions of retina, i.e. the neuroblastic layer progenitors (NB), ciliary body progenitors (CB), and optic nerve head glial progenitors (ONH) (Fig. 3.4a). Remarkably, Sox2 is also expressed homogeneously and at an overall stronger level within the Zic2\(^+\) VT RGC zone and colocalizes with Zic2 and Isl1/2 expression. Sox2 is completely absent from DT RGCs, indicated by Isl1/2 expression, including the most peripheral cells. Thus, Sox2 mRNA expression is maintained in postmitotic Zic2\(^+\) RGCs and, similar to SERT, is a very clean marker of this RGC subset (Fig. 3-2).
The transcription factor Math5 is expressed in retinal progenitors after they acquire competence to generate RGCs (Wang et al., 2001, Brzezinski et al., 2012) and thus marks the first neurogenic competence state of retinal progenitors (Yang et al., 2003). Similar to Sox2, Math5 mRNA expression extends into the Zic2⁺ RGC zone. However, unlike the homogeneous expression of Sox2, Math5 co-localizes with Zic2 expression in peripheral RGCs (Fig. 3.4b, red arrows indicate Zic2⁺ nucleus in red with surrounding Math5 mRNA in black). This co-localization is not as pronounced in RGCs of DT retina. Four additional genes that are expressed in various progenitor zones within the retina and also at lower levels in VT RGCs are gap junction protein Gja1, also known as connexin 43 (in CB and ONH), transcription factors Lhx2 (CB and NB) and Zic1 (CB), and cyclin D2 (CB) (Fig. 3.5). Gja1 and Lhx2 expression is not detectable in VT RGCs at E15.5, but is slightly higher at E14.5, suggesting that these genes are downregulated within this zone as the retina matures.

The G1 cyclin, cyclin D2 (Ccnd2) is strongly expressed in the proliferating cells peripheral to the RGC zone; Ccnd2 mRNA appears to localize basally within these cells. Basally localized Ccnd2 mRNA can also be detected at a lower level within the entire span of the Zic2⁺ RGC zone (Fig. 3.5d). The role of Ccnd2 in cell cycle regulation and its relationship to fate determination in the CNS will be further explored in Chapter 5.

To show more definitive co-localization of Sox2 and Zic2 expression, I co-immunostained E14.5-E16.5 retina for Zic2, Isl1/2, and Sox2 with a well-documented commercially available antibody (Santa Cruz) (Georgi and Reh, 2010). However, Sox2 protein expression is mostly absent in RGCs marked by Isl1/2, including Zic2⁺ RGCs (Fig. 3.6). Following a similar pattern seen before, Sox2 weakly co-localizes with RGCs located more peripherally and apically within the VT retina, suggesting that Sox2 is post-transcriptionally
regulated within VT retina, such that all Zic2⁺ RGCs express Sox2 mRNA, but only a small subset express Sox2 protein. These findings suggest that Sox2 is less likely to play a functional role within ipsilateral RGCs, but hint at an even more complicated upstream regulatory program functioning within these cells. I was unable to determine whether Math5 has a similar discrepancy in mRNA and protein expression in ipsilateral RGCs, as there are no reliable Math5 antibodies available.

In summary, Sox2 and Math5 expression patterns indicate that ipsilateral RGCs retain the expression of genes initially expressed prior to differentiation. This may be due to an upstream regulatory program that differs between ipsilateral and contralateral RGCs, turning off the expression of progenitor genes within contralateral RGCs but allowing for their continued expression in ipsilateral RGCs. These results raise the intriguing question of why Zic2⁺ ipsilaterally projecting RGCs maintain the expression of such genes.

**Genes that are expressed in all RGCs**

While the gene candidates described in the previous two sections represent new markers of ipsilateral and contralateral identity, a number of other candidates I explored via expression analysis in retinal sections were not differentially expressed between these two cell populations (Fig. 3.7). However, three of the contralateral RGC-enriched gene candidates that I analyzed were expressed uniformly in all RGCs and have not been previously described in the neural retina. The first of these genes is part of the membrane-anchored semaphorin guidance protein family, Sema7a, a growth promoting semaphorin that enhances central and peripheral axon growth (Pasterkamp et al., 2003). The zinc transporter LIV-1 of Zip6 (Slc39a6) has mostly been studied in the context of several cancers in which it is upregulated and thought to promote
epithelial-mesenchymal transitions (Grattan and Freake, 2012). A recent study suggested that LIV-1 is a target of growth factor signaling (Lue et al., 2011). Whether this gene has similar links to growth factor signaling and extracellular matrix interactions during development, especially of RGCs, is completely unknown. Lastly, N-ethylmaleimide-sensitive factor attachment protein, beta (Napb), also known as SNAP-beta, is part of the alpha/beta-NSF complex that recycles SNARE complex proteins after synaptic vesicle fusion (Sudhof, 2004, Wojcik and Brose, 2007, Burgalossi et al., 2010). Thus, each of these RGC-expressed genes is predicted to play a role in RGC development at later stages and may have interesting functions to explore.

**Discussion**

The increasing availability of genomic technologies within the last decade has facilitated high throughput identification of genes that define distinct cell types. By using the retrograde labeling and cell sorting method I developed for isolating pure populations of ipsilateral and contralateral RGC populations during embryonic development, I was able to use DNA microarray analysis to investigate the molecular identity of these two RGC subtypes. These gene profiling experiments uncovered a number of genes that are differentially expressed in ipsilateral and contralateral RGCs and were subsequently confirmed by endogenous expression in developing retina, thus having high biological relevance.

In particular, the new contralateral RGC markers that I identified fill a significant void, as most genes that have been described as important for contralateral RGC function are not exclusively expressed in these cell types. My microarray screen also identified genes that are expressed in ipsilateral but not contralateral RGCs. The shared expression of many of these
genes in both ipsilateral RGCs and cells within proliferative zones of the retina suggests that the mechanisms regulating differentiation for ipsilateral and contralateral RGCs are quite different. Finally, my gene profiling results also yielded RGC-specific genes that have not be previously described in the retina.

The developmental roles of all of these genes will only be revealed through further analysis by gain- and loss-of-function experiments, either through the use of mutant mouse models or overexpression and knockdown studies by *in utero* and *ex vivo* electroporation (Petros et al., 2009a). However, these gene profiling and expression studies themselves provide a number of interesting perspectives on the development of ipsilateral and contralateral RGCs.

**Ipsilateral and contralateral RGCs are molecularly distinct**

Recent single cell gene profiling experiments have revealed that even individual RGCs and retinal progenitors are heterogeneous in their expression profile (Trimarchi et al., 2008a, Trimarchi et al., 2008b). My gene profiling experiments reveal that ipsilateral and contralateral RGCs have distinct molecular signatures and can be distinguished during development not only by their axon guidance programs but also by many genes that play diverse functions in growth, differentiation, and fate specification. Additional studies using similar retrograde labeling and cell sorting approaches to isolate ipsilateral and contralateral RGCs in mature retina can provide further insight into how these two cell types are molecularly and functionally distinct beyond development.
**Ipsilateral RGCs are developmentally less mature than contralateral RGCs**

One highlight of my microarray results is that ipsilateral RGCs exhibit expression levels of various transcription factors normally expressed in retinal progenitor cells, as compared to contralateral RGCs (Figs. 3.4 and 3.5). Two possible explanations for these observations are: 1) Zic2\(^+\) RGCs are developmentally less mature than their dorsal counterparts, and failure to downregulate these genes reflect their immaturity; 2) these progenitor cell markers have additional functions in postmitotic Zic2\(^+\) RGCs.

Expression of progenitor genes in ipsilateral RGCs can be caused by perdurance of progenitor mRNAs in postmitotic RGCs and may simply reflect the relative neoteny of ipsilateral versus contralateral RGCs. Alternatively, this expression may suggest that ipsilateral RGCs derive from a progenitor pool at a different competence stage. Thus, the temporal control of neuronal differentiation may be a differential feature or regulator of ipsilateral versus contralateral RGC fate. In this vein, the same VT region that gives rise to ipsilateral RGCs during earlier retinal growth switches to generating contralaterally projecting RGCs after E16. The importance of timing in controlling fate specification of cell class in the retina (Livesey and Cepko, 2001) and elsewhere in the CNS (Caviness et al., 2003), as well as divergent projections of the same subclass (Imamura et al., 2011), supports this hypothesis.

One potential mechanism for controlling the timing of cell cycle exit and differentiation is through differential expression of cell cycle regulators (Dyer and Cepko, 2001, Ross, 2011). Thus, the finding that cyclin D2 is expressed within the VT RGC zone is particularly interesting. The G1 cyclin throughout most of retinal development is cyclin D1, and cyclin D1 null mice have severely hypocellular retinas (Sicinski et al., 1995). While the expression of cyclin D2 in
the peripheral margin of the retina has been previously shown as part of large-scale expression analyses (Glickstein et al., 2007a, Trimarchi et al., 2009), the cyclin D2 null mouse lacks a gross retinal phenotype similar to the microophthalmia present in the cyclinD1 null (Sicinski et al., 1996). Studies in cortical, cerebellar, and spinal cord development, on the other hand, have demonstrated a cell-type specific dependence on the different cyclin D family members, in particular cyclin D1 versus D2 (Huard et al., 1999, Lukaszewicz and Anderson, 2011, Ross, 2011). Thus, the distinct expression patterns of cyclin D1 and D2 in retinal development, and the close juxtaposition of cyclin D2 with the highly specialized subpopulation of RGCs projecting ipsilaterally, hint at a potential function of cyclin D2 expression in ipsilateral RGC production. I will present a more detailed spatiotemporal expression analysis and functional studies of cyclin D2 in embryonic retina in the following chapter.

Additionally, ipsilateral RGC-enriched developmental genes may also play a role in ipsilateral RGC identity and function. Indeed, several recent studies suggest that neural progenitor genes also have distinct functional roles in postmitotic neurons. Expression of early eye field transcription factors has been shown in both retinal progenitors and differentiated retinal cell types. For example, Pax6 is expressed in both retinal progenitors and RGCs, and has a functional role in postmitotic RGC axonal guidance (Hsieh and Yang, 2009, Sebastian-Serrano et al., 2012). Similarly, while Sox2 is traditionally thought to maintain neural progenitor identity (Graham et al., 2003), it has also been demonstrated to play a role in neuronal differentiation (Cavallaro et al., 2008). In the retina, complete Sox2 ablation leads to dramatic loss of neural progenitors; however, reduction of Sox2 expression in hypomorphic or null compound heterozygotes leads to maturation defects specific to RGCs, while other cell types are mostly unaffected (Taranova et al., 2006).
**Ipsilateral and contralateral RGCs express different components of the Igf signaling pathway**

Another interesting set of genes detected by my gene profiling experiments includes components of the Igf signaling pathway. Igf1 is enriched in contralateral RGCs (Table 3.4 and Fig. 3.4), whereas Igfbp5 is enriched in ipsilateral RGCs. Further suggesting the complementary expression pattern of these two genes, the most peripheral DT RGCs that lack Igf1 expression, express Igfbp5. However, because this observation was made correlatively by comparing ISH for these two genes on different retinal sections, definitive demonstration of mutually exclusive expression would require double-ISH within the same sections over different developmental time points. While Igfbp5 is the only Igf binding protein detected by this microarray study, another Igf peptide, Igf2, was enriched in the ipsilateral RGC population. Igf2 has been previously reported as a gene preferentially expressed in peripheral mouse retina (Trimarchi et al., 2009). Thus, it would be interesting to conduct detailed analysis of Igf2 signaling with ipsilateral and pan-RGC markers to determine whether Igf2 is indeed expressed in ipsilateral RGCs, whether it is absent from contralateral RGCs, and whether its expression overlaps with or is distinct from Igf1 and Igfbp5.

Together, these findings raise the question of how Igf signaling components are restricted to distinct cell populations within the retina and what the functional significance of this differential expression is. Igf binding proteins have a high affinity for Igfs and potently modulate the interactions of Igfs with their receptors, both positively and negatively, primarily through regulating the bioavailability of these peptides (Fernandez and Torres-Alemán, 2012). That Igfs affect proliferation, survival, and differentiation of all regions of the CNS has been long known (D'Ercole et al., 1996). More recently, Igf1 has also been implicated in neuronal circuitry formation by promoting axon outgrowth in corticospinal motor neurons (Ozdinler and
Macklis, 2006) and by acting as a chemoattractant that directs olfactory neuron axons to innervate the lateral olfactory bulb (Scolnick et al., 2008). Insulin receptor signaling has also been implicated in synapse maturation and density within the *Xenopus* retinotectal circuit (Chiu et al., 2008). A different Igf binding protein, Igfbp4 is expressed in CSMN neurons as well (Arlotta et al., 2005), and is under area-specific control by COUP-TFI (Tomassy et al., 2010). With its expression of multiple Igf signaling pathway proteins in distinct RGC populations, the developing retina offers an excellent opportunity to study the specific requirements for these genes in neuronal development.
Figure 3.1. Microarray analysis of ipsilateral and contralateral RGCs purified at E16 reveals distinct expression profiles.

A) qRT-PCR analysis of Zic2 and SERT expression was used to assess purity of retrogradely labeled, FACS purified samples of ipsilateral and contralateral RGC samples. Representative qPCR results of one set of samples are shown. Samples with robust enrichment of Zic2 and SERT in the ipsilateral RGC population were selected for gene profiling. B) Microarray analysis reveals 299 and 40 unique genes at least 2-fold increased in ipsilateral or contralateral RGCs, respectively ($p \leq 0.05$, Benjamin-Hochberg correction). C) The majority of the differentially expressed genes were upregulated in ipsilateral RGCs.
Figure 3.1. Microarray analysis of ipsilateral and contralateral RGCs purified at E16 reveals distinct expression profiles.
Table 3.1. Gene ontology analysis of genes differentially expressed in ipsilaterally and contralaterally projecting RGCs.

Genes that showed $\geq 2$-fold enrichment in ipsilateral or contralateral RGCs (corrected p-value $< 0.05$) were analyzed by GeneSpring GX (GO analysis) for functional groups. Most highly represented biological processes confirm that differentially expressed genes have developmental functions.

**Highly represented GO groups:**

- developmental process
- regulation of metabolic process
- multicellular organismal development
- regulation of gene expression
- anatomical structure development
- system development
- cell cycle
- cellular developmental process
- cell differentiation
- organelle organization
- nervous system development
- organ development
- cell division
- regulation of cell proliferation
<table>
<thead>
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<th>qPCR</th>
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<td>FC</td>
<td>p-value</td>
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<td>Gja1</td>
<td>gap junction protein, alpha 1 (connexin 43)</td>
<td>17.60</td>
<td>0.001</td>
</tr>
<tr>
<td>Klf4</td>
<td>Kruppel-like factor 4</td>
<td>7.38</td>
<td>0.002</td>
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<td>Lhx2</td>
<td>LIM homeobox protein 2</td>
<td>4.72</td>
<td>0.009</td>
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<td>Otx2</td>
<td>orthodenticle homolog 2</td>
<td>5.02</td>
<td>0.021</td>
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<td>serotonin transporter (SERT)</td>
<td>4.49</td>
<td>0.004</td>
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<td>Sox2</td>
<td>SRY-box containing gene 2</td>
<td>7.47</td>
<td>0.029</td>
</tr>
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<td>Sparc</td>
<td>secreted acidic cysteine rich glycoprotein</td>
<td>12.47</td>
<td>0.000</td>
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<td>ZFP36</td>
<td>zinc finger protein 36</td>
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<td>0.002</td>
</tr>
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<td>Zic1</td>
<td>zinc finger protein of the cerebellum 1</td>
<td>5.67</td>
<td>0.002</td>
</tr>
<tr>
<td>Zic2</td>
<td>zinc finger protein of the cerebellum 2</td>
<td>5.78</td>
<td>0.016</td>
</tr>
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<td>Sema3E</td>
<td>semaphorin 3E</td>
<td>-2.20</td>
<td>0.003</td>
</tr>
<tr>
<td>Tbx20</td>
<td>T-box 20</td>
<td>-2.15</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Table 3.2. qRT-PCR validation of select microarray candidates shows inter-platform reproducibility.

Comparison of absolute fold-change (FC) values measured by microarray or qRT-PCR of select genes, including known ipsilateral RGC markers SERT and Zic2 (bold). Only 1 of 11 tested new candidates failed to show enrichment in the RGC population corresponding to microarray results (Otx2, red).
<table>
<thead>
<tr>
<th>High in Ipsi</th>
<th>Symbol</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulation of transcription</td>
<td>Math5</td>
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</tr>
<tr>
<td></td>
<td>Gli3</td>
<td>GLI-Kruppel family memer GLI3</td>
</tr>
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<td></td>
<td>Lhx2</td>
<td>LIM homeobox protein 2</td>
</tr>
<tr>
<td></td>
<td>Sox2</td>
<td>SRY-box containing gene 2</td>
</tr>
<tr>
<td></td>
<td>Sox9</td>
<td>SRY-box containing gene 9</td>
</tr>
<tr>
<td></td>
<td>Zic1</td>
<td>zinc finger protein of the cerebellum 1</td>
</tr>
<tr>
<td></td>
<td>Zic2</td>
<td>zinc finger protein of the cerebellum 2</td>
</tr>
<tr>
<td>Developmental signaling</td>
<td>Fzd5</td>
<td>frizzled homolog 5</td>
</tr>
<tr>
<td></td>
<td>Igfbp5</td>
<td>insulin-like growth factor binding protein 5</td>
</tr>
<tr>
<td></td>
<td>Ptch1</td>
<td>patched 1</td>
</tr>
<tr>
<td>Cell cycle regulation</td>
<td>Ccnd1</td>
<td>cyclin D1</td>
</tr>
<tr>
<td></td>
<td>Ccnd2</td>
<td>cyclin D2</td>
</tr>
<tr>
<td>Axon guidance</td>
<td>Sema4d</td>
<td>semaphorin 4D</td>
</tr>
<tr>
<td></td>
<td>Sema5b</td>
<td>semaphorin 5B</td>
</tr>
<tr>
<td>Other cellular functions</td>
<td>Gja1</td>
<td>gap junction protein, alpha 1 (connexin 43)</td>
</tr>
<tr>
<td></td>
<td>Slc6a4</td>
<td>serotonin transporter (SERT)</td>
</tr>
<tr>
<td></td>
<td>Sparc</td>
<td>secreted acid cystein rich glycoprotein</td>
</tr>
<tr>
<td>High in Contra</td>
<td>Regulation of transcription</td>
<td>Brna3</td>
</tr>
<tr>
<td></td>
<td>Tbx20</td>
<td>T-box 20</td>
</tr>
<tr>
<td>Signaling</td>
<td>Igf1</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>Axon guidance</td>
<td>Sema3e</td>
<td>semaphorin 3E</td>
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<td></td>
<td>Sema7a</td>
<td>semaphorin 7A</td>
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<tr>
<td>Other cellular functions</td>
<td>Napb</td>
<td>gap junction protein, alpha 1 (connexin 43)</td>
</tr>
<tr>
<td></td>
<td>Slc39a6</td>
<td>zinc transporter Zip6 or Liv-1</td>
</tr>
</tbody>
</table>

Table 3.3. Representative candidate genes categorized by biological function.

Gene candidates are categorized based on previously described biological function in published literature.
Figure 3.2. Expression patterns of known ipsilateral and contralateral RGC markers.

ISH analysis at E15.5 shows co-localization of Zic2 (A) and SERT (B) mRNA in Zic2⁺ ipsilateral RGCs in the VT retina. C) In contrast, IHC analysis shows complementary expression of contralateral RGC marker Brn3a and Zic2 in RGCs labeled with pan-Brn3 immunostaining. These expression patterns were used as standards for expression analysis of microarray gene candidates.
Figure 3.2. Expression patterns of known ipsilateral and contralateral RGC markers.
Figure 3.3. Expression patterns of genes enriched in contralateral RGCs.

ISH analysis at E15.5 show complementary expression of Tbx20 (A), Sema3e (B), and Igf1 mRNA and Zic2 (ipsilateral RGCs). Fgf12 is highly expressed in Zic2− RGCs with low levels of expression in Zic2+ cells. All candidate genes are expressed in Isl1/2+ RGCs.
Figure 3.3. Expression patterns of genes enriched in contralateral RGCs.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Microarray</th>
<th>ISH/IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pou4f1 (Brn3a)</td>
<td>Pou domain, class 4, TF1</td>
<td>-2.20</td>
<td>no</td>
</tr>
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<td></td>
<td></td>
<td>0.001</td>
<td>yes</td>
</tr>
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<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>no</td>
</tr>
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<td>Insulin-like growth factor 1</td>
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<td></td>
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<tr>
<td></td>
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</tr>
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<td>T-box 20</td>
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<td>central</td>
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<tr>
<td></td>
<td></td>
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<td>no</td>
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</table>

Table 3.4. Summary of expression profile for genes enriched in contralateral RGCs.

Expression analysis by ISH/IHC (Fig. 3.2c and Fig. 3.3) is summarized by localization of expression in ipsilateral and contralateral RGCs, the proliferative neuroblastic layer (NB) and ciliary body (CB), and optic nerve head glia (ONH). All genes were enriched in contralateral RGCs by microarray and confirmed by ISH.
Figure 3.4. Expression patterns of genes enriched in ipsilateral RGCs.

ISH analysis at E15.5.  A) Sox2 mRNA is expressed in neuroblastic layer and Zic2⁺ RGCs but not Zic2⁻Isl1/2⁺ RGCs.  B) Math5 mRNA is expressed in Zic2⁺ RGCs located in at the periphery of the RGC layer (red arrows).  C) Igfbp5 mRNA is expressed in a subset of Zic2⁺ RGCs in VT retina and a few Zic1⁻ RGCs in dorsal retina (red arrows).
Figure 3.4. Expression patterns of genes enriched in ipsilateral RGCs.
Figure 3.5. Expression patterns of genes expressed at low levels in ipsilateral RGCs.

ISH analysis at E14.5 and E15.5. A) Gja1 mRNA is expressed in the peripheral margin of the retina (CB) at E14.5 and E15.5 but also at low levels in the Zic2^+ RGC zone at E14.5. B) Lhx2 mRNA is expressed in the neuroblastic layer of the retina at E14.5 and E15.5 but also at low levels in the Zic2^+ RGC zone at E14.5. C) Zic1 mRNA and cyclin D2 mRNA are expressed in the peripheral margin of the retina and also at low levels in the Zic2^+ RGC zone at E15.5 (red arrows).
Figure 3.5. Expression patterns of genes expressed at low levels in ipsilateral RGCs.
Figure 3.6. Sox2 protein expression colocalizes with few Zic2⁺ RGCs.

IHC analysis at E15.5. Unlike Sox2 mRNA, Sox2 protein expression co-localizes with very few Zic2⁺ RGCs in VT retina.
Figure 3.6. Sox2 protein expression colocalizes with few Zic2^+ RGCs.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Microarray FC</th>
<th>P-value</th>
<th>ISH/IHC</th>
<th>Ipsi RGC</th>
<th>Contra RGC</th>
<th>NB</th>
<th>CB</th>
<th>ONH</th>
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<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
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<td>5.78</td>
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<td>weaker</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
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<td>no</td>
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<td>weak</td>
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</table>

Table 3.5. Summary of expression profile for genes enriched in ipsilateral RGCs.

Expression analysis by ISH (Fig. 3.2a-b, 3.4 and 3.5) is summarized by localization of expression in ipsilateral and contralateral RGCs, the proliferative neuroblastic layer (NB) and ciliary body (CB), and optic nerve head glia (ONH). Upper panel lists genes highly enriched in ipsilateral RGCs. Lower panel lists genes that are expressed in ipsilateral RGCs but at a lower level than other cell types in retina. All genes were enriched in ipsilateral RGCs by microarray.
Figure 3.7. Genes expressed in proliferative zones or all RGCs.

ISH analysis at E14.5 and E15.5. Fzd5 (A), Notch 1 (B), Sema5b (C), Sox9 (D), Sparc (E), and Zic3 (F) mRNA is expressed in proliferative zones of the retina but not ipsilateral RGCs. These genes were enriched in the ipsilateral RGC population by gene profiling. Napb (G), Sema7a (H), and Slc39a6 (I) are expressed in all RGCs and were enriched in the contralateral RGC population by gene profiling.
Figure 3.7. Genes expressed in proliferative zones or all RGCs.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Microarray</th>
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<tbody>
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<td>FC</td>
<td>p-value</td>
</tr>
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<td>Fzd5</td>
<td>gap junction protein, alpha 1 (connexin 43)</td>
<td>5.04</td>
<td>0.024</td>
</tr>
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<td>Notch1</td>
<td>Notch gene homolog 1</td>
<td>2.93</td>
<td>0.012</td>
</tr>
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<td>Sema5b</td>
<td>semaphorin 5B</td>
<td>4.11</td>
<td>0.007</td>
</tr>
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<td>Sox9</td>
<td>SRY-box containing gene 9</td>
<td>5.69</td>
<td>0.003</td>
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<td>Sparc</td>
<td>secreted acidic cysteine rich glycoprotein</td>
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<td>0.000</td>
</tr>
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<td>Slc39a6</td>
<td>Zinc transporter Zip6 or LIV-1</td>
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Table 3.6. Summary of genes expressed in proliferative zones or in all RGCS.

Expression analysis by ISH (Fig. 3.7) is summarized by localization of expression in ipsilateral and contralateral RGCs, the proliferative neuroblastic layer (NB) and ciliary body (CB), and optic nerve head glia (ONH). Upper panel lists genes enriched in ipsilateral RGCs by microarray but detected in proliferating cells by ISH. Lower panel lists genes enriched in contralateral RGCs by microarray but detected in all RGCs by ISH.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
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<th>ISH/IHC</th>
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<td>GLI-Kruppel family member GLI3</td>
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<td>0.015</td>
</tr>
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<td>Kruppel-like factor 4</td>
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<td>Patched homolog</td>
<td>2.16</td>
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<td>Smoothened</td>
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<td>semaphorin 4D</td>
<td>2.43</td>
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<td>Transforming growth factor beta 2</td>
<td>3.21</td>
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</table>

**Table 3.7. Summary of genes analyzed but not depicted.**

Expression analysis by ISH/IHC is summarized by localization of expression in ipsilateral and contralateral RGCs, the proliferative neuroblastic layer (NB) and ciliary body (CB), and optic nerve head glia (ONH). All listed genes were enriched in ipsilateral RGCs by microarray.
Chapter 4. A role for cyclin D2 in ipsilateral RGC production

Introduction

In the previous chapter, I identified a new cohort of genes that are differentially expressed in ipsilateral and contralateral RGCs. Further functional characterization of each of these individual genes in RGC development will shed light on how ipsilateral and contralateral RGCs diverge during development and the potential roles these genes play in other regions of the CNS. When considered collectively, a fascinating pattern emerges from my microarray results and subsequent expression analysis: ipsilateral RGCs maintain the expression of genes expressed in RGC precursors or retinal progenitor cells. These results suggest that ipsilateral RGCs are less mature than their contralateral counterparts, even when compared to the contralateral RGCs in the dorsal periphery.

However, work carried out by graduate student Punita Bhansali on the albino mouse retina reveals an alternative possibility. A peculiar phenotype of all genetic causes of albinism, a pigmentation disorder of skin and retinal pigment epithelium (RPE), is perturbed development of the visual pathways, including a decrease in the size of the ipsilateral RGC projections (Kaas, 2005). While it has long been known that there is a temporal lag in the maturation of the albino retina as compared to wildtype (Webster and Rowe, 1991, Ilia and Jeffery, 1996, 2000), Punita’s results demonstrated a delay in RGC production specifically within VT retina during the window of ipsilateral RGC production (Bhansali et al., in preparation). Although the mechanisms of RPE-neural retina interactions are still unknown, it is clear that the neurogenic programs specific to the VT retina are particularly sensitive to changes in those interactions.
Accumulating evidence suggests that ipsilateral RGC production follows a unique path, whether through a particular sensitivity to non-cell autonomous signals from the RPE or a different mechanism for downregulating progenitor cell-expressed genes during differentiation. Thus, I embraced the opportunity to use my microarray screen to identify genes acting in these pathways. As I alluded in earlier chapters, one mechanism for controlling the timing of cell cycle exit and differentiation is through differential expression of cell cycle regulators (Dyer and Cepko, 2001, Ross, 2011). Thus, I was intrigued to find a number of cell cycle regulators enriched in ipsilateral RGCs in my microarray data.

In particular, cyclin D2 stood out as an interesting candidate, as its function has not been previously examined in the retina due to the lack of a gross retinal phenotype in cyclin D2 null mice (Sicinski et al., 1996). In contrast, mice that lack the related cyclin D1 have severely hypoplastic retinas (Fantl et al., 1995, Sicinski et al., 1995), and the role of cyclin D1 as the predominant G1 cyclin during retinal development has been extensively studied (Dyer and Cepko, 2001, Geng et al., 2001, Das et al., 2009). However, numerous studies within the last decade have revealed a tissue and even cell-type specific requirement for different cyclin D family members for cell proliferation and differentiation during development (Huard et al., 1999, Ciemerych et al., 2002, Glickstein et al., 2007b, Glickstein et al., 2009, Lukaszewicz and Anderson, 2011). Given the highly restricted expression of cyclin D2 in the retina (Glickstein et al., 2007a, Trimarchi et al., 2009) previous analyses of the cyclin D2 mutant may have missed a cell-type specific impact of cyclin D2 loss on a small population of cells within the retina, such as the ipsilateral RGCs.

As part of the expression analysis I performed to validate my microarray screen, I found that cyclin D2 is asymmetrically expressed within the retina, with a higher level of expression in
the ventral periphery, close to the ipsilateral RGC domain (Fig. 3.5D). This domain of the mouse retina will eventually develop into the ciliary body. Coincidentally, the ventral periphery is the source of the Zic2-expressing ipsilateral RGCs that arise during *Xenopus* metamorphosis, when binocular vision develops (Marsh-Armstrong et al., 1999). However, the contribution of proliferating cells within the retinal periphery during embryonic development, on other hand, has not been studied in detail. Given the expression of cyclin D2 within the periphery and its established role in the production of specific neuronal subtypes elsewhere in the CNS, I wondered whether cyclin D2 marks a distinct neurogenic zone within the ventral periphery of the retina that contributes to the production of ipsilateral RGCs.

In this chapter, I address this question through a careful analysis of cyclin D2 expression throughout embryonic retinal development and a characterization of the cyclin D2 null mouse in the context of ipsilateral versus contralateral RGC production.

**Results**

*Cyclin D2 is selectively expressed in the ventral periphery during the window of ipsilateral RGC production*

In the previous chapter, I demonstrated by ISH that cyclin D2 is expressed asymmetrically in the peripheral retina at E15.5, with a higher level and broader zone of expression in ventral retina as opposed to dorsal retina (Fig. 3-5d). Moreover, lower levels of cyclin D2 expression extend into the Zic2+ RGC zone. However, cyclin D2 mRNA appears concentrated at the basal side of the retina, suggesting that it is restricted to a specific compartment within these cells rather than distributed throughout the cytoplasm. This cellular
expression pattern also makes it difficult to determine whether cyclin D2 mRNA and Zic2 protein expression co-localize in the same cells. To determine where cyclin D2 protein localizes, I immunostained E15.5 retinal sections with antibodies against cyclin D2, Zic2 (ipsilateral RGCs), and Brn3 (all RGCs). Consistent with the ISH results, IHC analysis showed that cyclin D2 is expressed within the retinal periphery and the optic nerve head, and is substantially higher in ventral than dorsal periphery (Fig. 4.1). However, immunostaining revealed that cyclin D2 protein is nuclear rather than at the basal surface, with cells expressing highest levels of cyclin D2 positioned more basally. This expression pattern has been similarly reported in cortex (Tsunekawa and Osumi, 2012). Cyclin D2+ cells that showed positive staining for Zic2 and Isl1/2 were very rare, indicating that cyclin D2 is predominantly in cells that are situated in the periphery of VT retina, adjacent to the zone of Zic2-expressing cells, and do not yet express markers of differentiated RGCs such as Brn3 or Isl1/2.

In contrast to cyclin D2, cyclin D1 expression is homogeneous throughout proliferating regions in the retina (Fig. 4.2). Cyclin D2+ cells are also positive for cyclin D1. The broad expression of cyclin D1 is consistent with its role as the major D cyclin in retinal development required for general retinal histogenesis (Fantl et al., 1995, Sicinski et al., 1995). The additional expression of cyclin D2 in a specific subset of cells within the retina, however, suggests that it conveys an added layer of regulation on the cell cycle kinetics of these cells.

The expression of the ipsilateral RGC marker Zic2 peaks between E14 and E16 (Herrera et al., 2003a), and recent birthdating studies in our lab suggest that the peak production of these RGCs occurs between E13 and E15 (C. Soares, P. Bhansali, and F. Marcucci, unpublished). Given my hypothesis that cyclin D2 is enriched in ipsilateral RGC progenitors or precursors, I would expect asymmetric expression of cyclin D2 within the peripheral retina to correspond to
and precede the window of ipsilateral RGC production. Indeed, co-immunostaining for cyclin D2 and Brn3 revealed that cyclin D2 is expressed in neural retina as early as E11.5 (Fig. 4.3d) and is highly expressed in the ventral periphery from E11.5 to E14.5, as compared to the dorsal periphery (Fig. 4.3, red arrows). Interestingly, at E13.5 and E14.5, cyclin D2$^+$ cells intermingle with Brn3$^+$ RGCs (Fig. 4.3 a-b, yellow arrows) within ventral but not dorsal retina, where the two expression domains have distinct boundaries. This overlap in the cyclin D2 and RGC domains raises the possibility that the Ccnd2$^+$ cells within this overlapping domain are in the process of differentiating into Brn3$^+$ RGCs.

While the cyclin D2-expressing domain is immediately adjacent to the Brn3$^+$ RGC domain from E12.5-E15.5, it is interesting to note that a large gap separates these two domains at E11.5 (Fig. 4.3d). As noted by the small number of Brn3$^+$ RGCs present within the central retina at this age, the central-to-peripheral wave of neurogenesis has just commenced within the retina, and thus the cells separating the Brn3 and cyclin D2 domains are likely to be proliferating neural progenitors at the outskirts of this wave. This pattern raises the important point that the cyclin D2 domain is not always adjacent to the peripheral edge of neurogenesis, in which case it could mark an intermediate progenitor state, as seen in the cortex (Glickstein et al., 2009). Instead, cyclin D2 expression appears to be consistently restricted to a distinct domain in the peripheral retina, with a strong ventral bias at early stages of retinal development.

What then happens to cyclin D2 expression after the peak period of ipsilateral RGC production? Does the asymmetry in cyclin D2 expression persist in the peripheral retina? Immunostaining at E16.5 revealed that cyclin D2 expression within the peripheral retina is reduced and appears to be mostly symmetric between dorsal and ventral retina (Fig. 4.4). Thus, the ventral-high expression of cyclin D2 only persists until E15, and both precedes and overlaps
the window of ipsilateral RGC production. Thus, the spatiotemporal pattern of cyclin D2 expression supports the hypothesis that this particular D-type cyclin is expressed in ipsilateral RGC progenitors.

**Loss of cyclin D2 results in a reduction in Zic2⁺ RGCs at early embryonic ages**

If cyclin D2 is in fact expressed in ipsilateral RGC progenitors and important for their normal cell cycle progression, then cyclin D2 deficiency should alter the cell cycle parameters of these cells and consequently may affect Zic2⁺ RGC production. As a readout for this process, I first examined whether the number of Zic2⁺ RGCs are altered in Ccnd2⁻/⁻ mice at E15.5, the peak period of Zic2⁺ expression in RGCs. Immunostaining for Zic2 in E15.5 retinal sections revealed that Zic2⁺ RGCs are visibly reduced in Ccnd2⁻/⁻ embryos as compared to their Ccnd2⁺/⁺ littermates (Fig. 4.5a). Quantification of the total number of Zic2⁺ RGCs from every third retinal section through the entire eye showed a 37.5% overall reduction in Ccnd2⁻/⁻ retinas (Fig. 4.5b). Although this reduction is not statistically significant ($p=0.093$) in the small sample size examined ($n=2$ for each phenotype), I expect the reduction to become statistically significant with a larger sample size due to the conspicuous nature of the phenotype. While retinal area appears to be slightly decreased in the Ccnd2⁻/⁻ mutant, this result is not statistically significant (Fig. 4.5c). Moreover, the decrease in Zic2⁺ RGCs is much more dramatic, suggesting that loss of cyclin D2 has a subtype-specific effect on RGC production.

One mechanism through which cyclin D2 may regulate ipsilateral RGC production is through the regulation of cell cycle exit in progenitor cells. In cortical progenitors, cyclin D2 loss is hypothesized to promote premature cell cycle exit and neuronal differentiation by lengthening G1 (Glickstein et al., 2009). To determine how cyclin D2 affects the time course of
ipsilateral RGC production, I next assessed the number of Zic2+ RGCs in Ccnd2+/+ versus Ccnd2−/− embryos at three additional developmental time points: E13.5 (prior to Zic2 expression), E14.5 (beginning of Zic2 expression in RGCs), and E17.5 (end of peak period of Zic2 expression).

Analysis at E13.5 showed no precocious expression of Zic2 in E13.5 Ccnd2−/− RGCs (data not shown), demonstrating that loss of cyclin D2 does not lead to earlier production of Zic2+ RGCs. At E14.5, Zic2+ RGCs in Ccnd2−/− retina (n=2) are even more dramatically reduced than at E15.5 (Fig. 4.6a), with a 63.4% decrease (p=0.022) in the total Zic2+ RGC number relative to Ccnd2+/+ (n=2) (Fig. 4.6b) that is not accompanied by a comparable reduction in retinal size (Fig. 4.6c). Moreover, the overall distribution of Zic2+ RGCs is similar between the two genotypes (Fig. 4.6d). In contrast, Zic2+ RGC numbers are similar between Ccnd2+/+ (n=1) and Ccnd2−/− retina (n=2) at E17.5 (Fig. 4.7) suggesting that ipsilateral RGC production recovers towards the end of its developmental window. Given that the findings that I have presented are still preliminary, these experiments need to be carried out with a larger sample sizes and rigorous statistical testing.

The pattern of Zic2+ RGC reduction at early but not late developmental stages is reminiscent of another mutant, the albino (Tyr(−2)/−2j) mouse, where the decrease in Zic2+ RGCs is the consequence of an overall delay in RGC production within VT retina (Bhansali et al., in preparation). To determine whether loss of cyclin D2 similarly affects RGC production within VT retina, I analyzed the number of differentiated RGCs as marked by Isl1/2 staining within a 200 µm section from the peripheral boundary of RGCs in two matched Ccnd2+/+ and Ccnd2−/− temporal retinal sections (Fig. 4.6d, circled sections). These sections were selected because they show the greatest reduction in Zic2+ RGC number. However, unlike the albino VT retina,
Ccnd2−/− VT retina does not contain a decreased total number of RGCs; in fact, the Isl1/2+ RGC count is slightly increased in Ccnd2−/− VT retina as compared to Ccnd2+/+, though not statistically significant (Fig. 4.6e). Thus, although the albino and Ccnd2−/− mutants show a similar temporal pattern of Zic2+ RGC reduction, this reduction may be caused by changes in differing mechanisms that influence ipsilateral RGC production or fate specification. Moreover, preliminary analysis suggests that cyclin D2 expression is not altered in the albino retina (data not shown).

The reduction in Zic2+ RGCs cyclin D2−/− mutants corresponds to a reduced ipsilateral RGC projection at E17.5

The results I have presented thus far suggest that fewer ipsilateral RGCs are found in Ccnd2−/− retina at E14.5 and E15.5, as seen by the reduction in Zic2 expression in RGCs. However, to ensure that this reduction reflects a true loss of ipsilateral RGC identity and not merely a spatiotemporal decrease in Zic2 expression, analysis of downstream markers of ipsilateral RGC identity is required. One such gene, SERT, is expressed in ipsilateral RGCs and plays a role in the refinement of ipsilateral RGC axons within the dLGN at postnatal ages (García-Frigola and Herrera, 2010). Analysis at E14.5 showed a decrease in SERT expression within VT RGCs of Ccnd2−/− retinas compared to Ccnd2+/+ that is consistent with the reduction in the number of cells expressing Zic2 observed in the mutants (Fig. 4.8). Moreover, anterograde Dil labeling to visualize the axonal projections from a single retina (Fig. 4.9), showed a significant reduction (p=0.002) in ipsilaterally projecting retinal axons in E17.5 Ccnd2−/− embryos (n=7) as compared to Ccnd2+/+ (n=3) (Fig. 4.9e). In contrast, contralateral RGC axons are not significantly reduced (Fig. 4.9d). Preliminary analysis of retinal projections in P0 mutant and
wild-type pups also shows a decrease in the size of the ipsilateral projection, although this reduction appears less dramatic (Fig. 4.9e), possibly reflecting the recovery of Zic2+ RGC number seen at E17.5 or caused by a developmental delay in ipsilateral RGC axon outgrowth. Together, these results confirm that the reduction in Zic2+ RGC number at E14.5 and E15.5 is correlated with a reduction in an additional ipsilateral RGC marker, SERT, as well as the number of RGC axons that project ipsilaterally, thus strengthening the argument that ipsilateral RGCs are indeed reduced in the Ccnd2−/− retina.

**Discussion**

The G1 phase of cell cycle presents an opportunity for extracellular signals to influence a cell’s decision to divide, through the action of the three D-type cyclins (Massagué, 2004). It is now increasingly apparent that cyclin Ds play an influential role in cell fate decisions and controlling the production of specific cell types through their distinct expression patterns during CNS development and cell-type specific requirements for these genes (Huard et al., 1999, Lukaszewicz and Anderson, 2011, Ross, 2011). In this chapter, I have explored the role of one of these genes, cyclin D2, in the development of the retina, where it has not previously been thought to play an essential role (Sicinski et al., 1996).

Through detailed spatiotemporal analysis of its expression pattern during retinal development, I have discovered that cyclin D2 is asymmetrically expressed in a proliferative compartment whose contribution to embryonic retinal development is unclear: the developing ciliary body within the retinal periphery. Within this compartment, cyclin D2 is more highly expressed in ventral than in dorsal retina during a time window preceding and coinciding with the production of ipsilateral RGCs (Figs. 4.1-4.4). Thus, the expression pattern of cyclin D2
suggests that it is expressed in a specific population of retinal progenitors that may contribute to ipsilateral RGC production. I further demonstrate that ipsilateral RGCs are reduced in the cyclin D2 null mutant, both through careful characterization of ipsilateral RGC markers as well as anatomical analysis of the decussating retinal axon projections.

Through this combination of expression and functional analyses, I have thus demonstrated a novel role for cyclin D2 expression in controlling ipsilateral RGC production during embryonic development. In particular, these results raise several fascinating questions regarding both the significance of the retinal periphery in mammalian retinal development and how cyclin D2 exerts its control on these progenitors.

**Cyclin D2 is expressed in a distinct population of proliferative cells within the peripheral retina during embryonic development**

A fascinating observation is that cyclin D2 is expressed within the peripheral margin of the retina, a region that in mammals, develops into the ciliary body and other anterior segment structures of the eye (Chow and Lang, 2001, Napier and Kidson, 2007). In fish and amphibians, a neurogenic region is maintained between the ciliary epithelium and neural retina, the ciliary margin zone (CMZ), which acts as a continuous source of neurogenesis in adulthood (Kubota et al., 2002). In animals that have significant growth of the eye after embryonic and neonatal development, the CMZ can produce more retinal cells than are produced during embryonic histogenesis, behaving like a true stem cell compartment and generating all types of retinal neurons and glia (Wetts et al., 1989).
Although mammals do not appear to have an equivalent CMZ, recent studies in mice have shown that the retinal margin can be coaxed into retaining a small number of proliferating cells into adulthood on a *Patched* haploinsufficiency background (Moshiri and Reh, 2004). The contribution of proliferating cells within the mouse retinal periphery to embryonic RGC development, on other hand, has not been studied in detail. A number of the genes I found upregulated in ipsilateral RGCs were also expressed in the peripheral retinal margin (Lhx2, Gja1, Zic1, Cyclin D2, Fig. 3.5) or in the proliferative zone immediately peripheral to the RGCs (Math5, Sox2, Fig. 3.4). The expression of Sox2 in this region is substantially more pronounced in VT periphery than in the dorsal retina, extending well into to the retinal periphery beyond the Isl1/2 boundary (Fig. 3.4a, black arrow). These findings raise the provocative question of whether a distinct neurogenic zone within the ventral periphery of the retina, much like the postnatal CMZ of lower vertebrates, contributes to the production of a specific RGC subtype, the ipsilateral RGC population.

**Does the ventral retinal periphery contain a distinct progenitor pool for ipsilateral RGCs?**

In *Xenopus*, an ipsilateral RGC projection does not develop embryonically but rather during metarmorphosis, when the eyes move frontally and binocular vision is established (Hoskins and Grobstein, 1985b). During this critical developmental window, the ventral CMZ is the source of the Zic2-expressing ipsilateral RGCs that are produced and specifically proliferates in response to thyroid hormone (Hoskins, 1986). In contrast, the dorsal CMZ is unresponsive to these stimulatory effects due to its expression of the thyroid hormone inactivator type III deiodinase (Marsh-Armstrong et al., 1999). Thus, this asymmetric behavior of the dorsal and ventral retinal periphery in *Xenopus* corresponds to the asymmetric expression of cyclin D2 I
have observed in the retinal periphery of mouse retina during the window of ipsilateral RGC development. Importantly, asymmetry in cyclin D2 expression is only apparent from E11-E15, before or during the period when the majority of ipsilateral RGCs are produced (Fig. 4.1, 4.3), and no longer at E16, when ipsilateral RGC production wanes (Fig. 4.4). Thus, the tightly regulated spatiotemporal expression pattern of cyclin D2 within the murine retina is highly suggestive of a population of cyclin D2-expressing progenitors that gives rise to ipsilateral RGCs.

Does the Xenopus retina, then, also express a cyclin D2 homolog within ventral CMZ during metamorphosis? While a cyclin D2 Xenopus homolog does exist, no studies have described its expression or function within the retia. However, a fascinating study identified a novel cyclin D, which the authors subsequently named ccndx, that is expressed in ventral neural structures, such as the neural tube, hindbrain, and olfactory region of Xenopus tropicalis (Chen et al., 2007). While this study focused on the requirement of ccndx for maintaining motor neuron progenitors, it also reported the collateral findings that ccndx is expressed in the ventral CMZ of X. tropicalis tadpoles and that Ccndx morphants display defects in ventral retina formation.

While early clonal analysis found that progeny of progenitors labeled with retroviral vectors are distributed in tightly organized radial columns (Turner and Cepko, 1987, Turner et al., 1990), later studies in chick and mouse showed that there is tangential migration of specific classes of retinal cells: cones, horizontal, amacrine, and ganglion cells (Fekete et al., 1994, Reese et al., 1995, Reese et al., 1999). Moreover, this tangential dispersion of cells is greater in peripheral than in central retina, and more likely to occur early in retinal development (Fekete et al., 1994). The group of Eloisa Herrera, a former postdoctoral fellow in the Mason lab who led the original studies on Zic2 control of the ipsilateral RGC projection (Herrera et al., 2003b), has
preliminary observations that support the idea of such tangential migration. By live imaging of organotypic slice cultures of embryonic retina from the Zic2-EGFP BAC transgenic mouse (described in Chapter 2), she and colleagues have witnessed GFP-positive cells from the ventral periphery that move centrally to populate the ipsilateral RGC zone (E. Herrera, personal communication). Whether the GFP$^+$ cells of ventral retinal periphery in Zic2-BAC transgenic mice are truly migrating tangentially into the RGC zone or simply adding on to the peripheral RGC zone, is still unclear. Moreover, it is unknown whether they express cyclin D2. Nevertheless, these observations further corroborate the hypothesis that the ventral periphery gives rise to ipsilateral RGCs.

To demonstrate that the cyclin D2-expressing cells of the murine ventral retinal periphery are a true progenitor source for ipsilateral RGC production, lineage-tracing experiments must be performed. A number of approaches can be used for performing these studies, although the ideal strategy would be to examine the lineage of cyclin D2-expressing cells in embryos bred from a Ccnd2-Cre and Rosa26-GFP or Rosa26-LacZ cross. However, as no Ccnd2-Cre lines are currently available, alternative approaches can be used in lieu of generating such a mouse. For example, lineage tracing can be performed using a mouse line with cre expression driven by a gene specifically expressed in the peripheral retina, such as Gja1 (Kretz et al., 2003), after confirmation that this gene co-localizes with cyclin D2 within the same cells.

Ipsilateral RGCs have a subtype-specific requirement for cyclin D2 activity

In the second half of my study, I functionally characterized the contribution of cyclin D2 to embryonic retinal development and found that ipsilateral RGCs are disproportionately affected in cyclin D2 null mice, demonstrated by a loss of ipsilateral RGC marker expression (Fig. 4.6-
and a decrease in ipsilaterally projecting retinal axons (Fig. 4.9). These results are the first demonstration of a requirement for cyclin D2 in retinal development. Moreover, this requirement appears to be subtype-specific. These findings are in line with studies in other areas of the CNS, where the different cyclin D family members have been shown to have cell-type specific effects on development, such as granule cells and stellate interneurons in the cerebellum (Huard et al., 1999) and parvalbumin (PV) interneurons in the cortex (Glickstein et al., 2007b).

Although ipsilateral RGC differentiation is disproportionately affected in the cyclin D2 null mouse, I cannot rule out the possibility that other RGC subtypes may also have some sensitivity to cyclin D2 loss. While I did not find a statistically significant decrease in the contralateral retinal projection at E17.5 (Fig. 4.9d), qualitative examination suggests that there is some decrease (Fig. 4.9c), especially at later developmental ages (Fig. 4.9f). This can be explained by the expression of cyclin D2 in the VN periphery during early development as well as dorsal periphery at later stages. These regions potentially harbor progenitors that produce contralateral RGCs, similar to ipsilateral RGCs that arise from the VT periphery. Thus, cyclin D2-expressing progenitors may have a ventrally biased contribution to RGC genesis only at early developmental ages, when cyclin D2 is mostly expressed in the ventral periphery, and these progeny include the ipsilaterally projecting RGCs. To explore these possibilities, further quantification of differentiated RGC numbers within the VN of Ccnd2+/+ and Ccnd2−/− at early ages, as well as all peripheral retinal regions at later ages, is needed. Moreover, to directly assess whether contralateral RGC numbers are affected, careful analysis of the numbers of contralateral RGCs within peripheral regions, as marked by Isl2 or Brn3a (or new contralateral markers identified in Chapter 3), is required.
Thus far, I have focused my analysis on RGC development. However, cyclin D2 may also have a subtype-specific effect on the development of other retinal cell classes. Apical progenitors throughout the neuroblastic layer of the retina progress through increasingly restricted competence states, during which they differentiate into various retinal cell classes (Livesey and Cepko, 2001). Thus, the cyclin D2-expressing progenitors of the retinal periphery may similarly contribute to the production of specific subpopulations of later-born cell-types within the peripheral retina.

*Cyclin D1 and D2 have distinct effects on retinal development*

Through co-immunostaining of retinal sections, I have demonstrated that while cyclin D1 expression is found in proliferating cells throughout the retina, cyclin D2 expression is superimposed on a select subset of these cells within the ventral retinal periphery, and to a much lesser extent, in the dorsal periphery (Fig. 4-2). Through histological analysis at both postnatal and embryonic ages, loss of cyclin D1 in mouse retina has been shown to cause a hypocellular phenotype that affects all retinal cell classes (Fantl et al., 1995, Sicinski et al., 1995, Ma et al., 1998, Das et al., 2009). In contrast, my results demonstrate that, at least within RGCs, specific subpopulations are differentially affected by cyclin D2 loss. Thus, while cells that have lost cyclin D2 expression in null mutants still express cyclin D1, the remaining cyclin D1 does not sufficiently compensate for the loss of cyclin D2 function in these cells, demonstrating non-redundant function of these two D cyclins within the retinal periphery.
Control of ipsilateral RGC production by cyclin D2

My results have demonstrated a link between cyclin D2 expression within a distinct progenitor zone in the retina and the production of ipsilateral RGCs. How then does cyclin D2 mediate this control? A number of different studies throughout the CNS have carefully dissected molecular mechanisms through which the D cyclins control proliferation and differentiation. The classic mechanism through which D cyclins are thought to influence fate decisions during neurogenesis is the control of G1 length, which in turn influences cell cycle exit decisions (Ross, 2011). The D cyclins are thought to shorten G1 length, thereby promoting entry into S-phase and delaying cell cycle exit. In the medial ganglionic eminence, for example, loss of Ccnd2 leads to G1 lengthening, resulting in cell cycle exit, premature terminal differentiation, and depletion of subventricular zone progenitors (Glickstein et al., 2007b). However, several recent studies have uncovered two cell-cycle independent roles for G1 cyclins: 1) direct transcriptional activation of Notch1 by cyclin D1 in retinal progenitors to promote proliferation (Bienvenu et al., 2010) and 2) conferral of apical progenitor fate in the cell that inherits cyclin D2 mRNA during an asymmetric neurogenic division (Tsunekawa et al., 2012).

The dual expression of cyclin D1 and cyclin D2 in a highly restricted progenitor zone during retinal development offers an excellent opportunity to study the differential contribution of these two cyclins to neurogenesis and fate acquisition of the select neuronal subtypes that are born within this region of the retina, as well as the mechanisms through which these two cyclins act. Does cyclin D2 cooperate with cyclin D1 to regulate G1 length? Or does it confer asymmetric fate acquisition in neuronal subtypes in a similar manner as other asymmetrically inherited genes, such as Notch in the spinal cord (Del Barrio et al., 2007, Peng et al., 2007)? Or does it act as a transcriptional activator of an ipsilateral RGC specification program containing or
upstream of Zic2? These are only some of the critical questions raised by the results of this chapter.
Figure 4.1. Cyclin D2 expression is greater in ventral peripheral retina than in dorsal at E15.5.

Coronal cryosections of E15.5 DT (A) and VT (B) retina immunostained with antibodies against cyclin D2, Zic2 (ipsilateral RGCs), and Brn3 (all RGCs). Cyclin D2 is expressed in the lens and the retinal marginal zone bordering RGCs (labeled with Brn3). Cyclin D2 is highly expressed in the ventral periphery (A) as compared to the dorsal periphery (D) (red arrows) and rarely co-localizes with Zic2 in RGCs.
Figure 4.1. Cyclin D2 expression is greater in ventral peripheral retina than in dorsal at E15.5.
Figure 4.2. Cyclin D1 and D2 have overlapping but distinct expression patterns.

Coronal cryosections of E14.5 DT (A) and VT (B) retina immunostained with antibodies against cyclin D1 and D2. Unlike the ventral-high expression of cyclin D2, cyclin D1 is expressed homogeneously in proliferating regions of the retina. Cell that express cyclin D2 also express cyclin D1, thus expression patterns of these cyclins is overlapping yet distinct.
Figure 4.2. Cyclin D1 and D2 have overlapping but distinct expression patterns.
Figure 4.3. Spatiotemporal changes in cyclin D2 expression in early RGC development (E11.5-E14.5).

Coronal cryosections of E14.5 (A), E13.5 (B), E12.5 (C), and E11.5 (D) retina immunostained with antibodies against cyclin D2 and Brn3 (RGCs). Cyclin D2 is expressed in the lens and the retinal marginal zone bordering RGCs (labeled with Brn3) from E12.5-E14.5. Cyclin D2 is highly expressed in the ventral periphery as compared to the dorsal periphery within neural retina (red arrows). At E13.5 and E14.5 (A-B), cyclin D2⁺ cells intermingle with Brn3⁺ RGCs only in ventral retina (yellow arrows) and have sharp boundaries in dorsal retina. The cyclin D2 and Brn3 domains are separated by a large gap at E11.5 (D).
Figure 4.3. Spatiotemporal changes in cyclin D2 expression in early RGC development (E11.5-E14.5).
Figure 4.3 (cont.). Spatiotemporal changes in cyclin D2 expression in early RGC development (E11.5-E14.5).
Figure 4.4. Cyclin D2 expression is no longer asymmetric at E16.5.

Coronal cryosections of E16.5 DT (A) and VT (B) retina immunostained with antibodies against cyclin D2, Zic2 (ipsilateral RGCs), and Brn3 (all RGCs). At E16.5, cyclin D2 expression is reduced in the retinal periphery and is no longer asymmetric in dorsal and ventral retinal (red arrows).
Figure 4.4. Cyclin D2 expression is no longer asymmetric at E16.5.
Figure 4.5. Zic2⁺ RGCs are reduced in Ccnd2⁻⁻ retinas at E15.5.

A) Matched coronal cryosections of E15.5 VT retina immunostained with antibodies against Zic2 (ipsilateral RGCs) and Brn3 (all RGCs) show visibly reduced numbers of Zic2⁺ RGCs in Ccnd2⁻⁻ embryos as compared to Ccnd2⁺⁺ littermates. B) The total number of Zic2⁺ RGCs is decreased by 36.5% in Ccnd2⁻⁻ embryos, although this reduction is not statistically significant (n=2, p=0.09). C) Retinal size in Ccnd2⁺⁺ and Ccnd2⁻⁻ embryos is not statistically different (n=2, p=0.06). Values are mean ± SEM.
Figure 4.5. Zic2+ RGCs are reduced in Ccnd2−/− retinas at E15.5.
Figure 4.6. Zic2⁺ RGCs are selectively reduced in Ccnd2⁻⁻ VT retina at E14.5.

A) Matched coronal cryosections of E14.5 VT retina immunostained with antibodies against Zic2 (ipsilateral RGCs) and Brn3 (all RGCs) show pronounced reduction in the numbers of Zic2⁺ RGCs in Ccnd2⁻⁻ embryos as compared to Ccnd2⁺⁺ littermates. B) The total number of Zic2⁺ RGCs is decreased by 65.4% ($p=0.02$) in Ccnd2⁻⁻ embryos (n=2) as compared to Ccnd2⁺⁺ (n=2) but not significantly decreased in Ccnd2⁺⁻ embryos (n=2, $p=0.13$). C) Zic2⁺ RGC count from nasal to temporal retina (every 3rd section): distribution and peak of Zic2 expression is similar in Ccnd2⁺⁺, Ccnd2⁺⁻, and Ccnd2⁻⁻ embryos. Zic2⁺ RGC reduction is greatest in temporal sections 7-9. D) Retinal size in Ccnd2⁺⁺ (n=1), Ccnd2⁺⁻ (n=2), and Ccnd2⁻⁻ (n=2) embryos is not statistically different ($p=0.653$). E) Isl1/2⁺ RGCs VT retina, measured in 2 matched sections (7-8) within a 0-200µm radial distance from the periphery are not significantly different between Ccnd2⁺⁺ (n=2) and Ccnd2⁻⁻ (n=2) retinas. Values are mean ± SEM. *$p<0.05$. 
Figure 4.6. Zic2⁺ RGCs are selectively reduced in Ccn2⁻/VT retina at E14.5.
Figure 4.6 (cont.). Zic2⁺ RGCs are selectively reduced in Ccnd2⁺VT retina at E14.5.
Figure 4.7. Zic2⁺ RGCs are no longer reduced in Ccnd2⁻/⁻ retina at E17.5.

A) Matched coronal cryosections of E17.5 VT retina immunostained with antibodies against Zic2 (ipsilateral RGCs) and Isl1/2 (all RGCs) in Ccnd2⁺/+ and Ccnd2⁻/⁻ littermates as compared to Ccnd2⁺/+ littermates. B) The total number of Zic2⁺ RGCs is similar in Ccnd2⁺/+ (n=1) and Ccnd2⁻/⁻ embryos (n=2). Values are mean ± SEM.
Figure 4.7. Zic2+ RGCs are no longer reduced in Ccnd2−/− retina at E17.5.
Figure 4.8. Ccnd2−/− mutants show decreased expression of ipsilateral marker SERT at E14.5.

Matched coronal cryosections of E14.5 VT retina. IHC shows reduced expression of SERT mRNA in Ccnd2−/− embryos as compared to Ccnd2+/+ littermates.
Figure 4.8. *Ccn2*<sup>−/−</sup> mutants show decreased expression of ipsilateral marker SERT at E14.5.
Figure 4.9. Ipsilateral RGC axons are reduced in the Ccnd2−/− optic tract at E17.5 and P0.

Whole-mounts of optic chiasm from E17.5 (C) and P0 (F) Ccnd2+/+ and Ccnd2−/− littermates.

Schema showing: A) Unilateral anterograde labeling of RGC axons with DiI from retina and visualization of optic chiasm; B) quantification of pixel intensities of contralateral and ipsilateral optic tracts (OTs). C) At 17.5, ipsilaterally projecting axons are visibly reduced in the ipsilateral OT of Ccnd2−/− mutants. Quantification of contralateral (D) and ipsilateral (E) OT intensities normalized to the Ccnd2+/+ average. The Ccnd2−/− (n=7) ipsilateral projection is significantly reduced by 23.8% (p=0.002) from Ccnd2+/+ (n=3). The ipsilateral projection of heterozygote mutants and the contralateral projections of both homozygous and heterozygous mutants are not significantly changed. F) At P0, ipsilaterally projecting axons appear to be reduced in the ipsilateral OT of Ccnd2−/− mutants but to a lesser extent than at E17.5. Values are mean ± SEM. **p<0.01.
Figure 4.9. Ipsilateral RGC axons are reduced in the $\text{Ccnd2}^{-/-}$ optic tract at E17.5 and P0.
Chapter 5. Discussion and Perspectives

Within the complex cellular network of the central nervous system, each group of related neurons is defined by a distinct molecular signature that confers upon them their unique morphology, connectivity, and functional purpose. These molecular signatures are also a record of their origin and the developmental pathways that shape their growth and maturation. One of the most breathtaking processes of development is how each one of these varied cell types arises from a single common ancestor: the fertilized oocyte that gives rise to all of the cells of the mature organism. However, developing cells are influenced by a plethora of signals, both pre-programmed and environmentally-derived, that shape the paths by which they diversify into their final, terminally differentiated state. Our understanding of the molecular cues that drive these processes has greatly expanded over the last few decades, as the role of transcription factors, cell cycle regulators, extrinsic signaling molecules, non-coding RNAs, and many other mechanisms have been dissected.

In the visual system of binocular animals, the ipsilaterally and contralaterally retinal ganglion cells are distinguished by the laterality of their axonal projections. While many of the axon guidance programs that direct the growth and decussation of these axonal pathways have been revealed over the last decade (Petros et al., 2008, Fabre et al., 2010, Erskine et al., 2011, Kuwajima et al., 2012), numerous gaps remain in our understanding of how these guidance molecules are transcriptionally regulated and how they are induced by the patterning genes that set up the different domains of the retina in which these RGC subtypes reside. An even more elusive question within the field is how the ipsilateral and contralateral RGC subpopulations acquire their different cell fates.
In my thesis project, I set out on a quest to dissect out the molecular signatures of the ipsilateral and contralateral RGC populations, with the hope that by understanding how these two RGC subtypes are different, I could uncover some clues to how they became different. The trajectory of my work has in fact followed the “retrograde labeling” approach that I utilized for purifying my cells of interest (Chapter 2). By using an output of a retinal ganglion cell’s identity—its axon trajectory—I traced two anatomically divergent subtypes of RGCs to the molecular signatures that define them at a developmental time point and most promisingly, have arrived at a potential difference in their origin within the embryonic retina.

Through gene expression profiling of purified ipsilateral and contralateral RGCs, I have uncovered the distinct molecular signatures that define and distinguish them during embryonic development. In Chapter 4, I presented one of these candidates, the G1 cell cycle regulator Ccnd2, which has taken us one step further to uncovering the mechanisms that differentiate ipsilateral and contralateral RGC specification and has opened up many new lines of speculation to how neuronal diversification is accomplished in the retina. Going forward, the functional characterization of additional validated gene candidates from this screen, such as Igfbp5, Igf1, Tbx20, Sema3e, and Fgf12 might also lead to discovery of novel regulatory pathways in ipsilateral and contralateral RGC development and function.

**Building neuronal subtypes through progenitors compartments: a common evolutionary strategy**

Comparative studies of retinal axon development in different model organisms have revealed both shared and distinct strategies retinal development. For example, in lower vertebrates such as fish and amphibians, neurogenesis within the retina continues throughout
adulthood as the eye continues to grow in size. As discussed in the previous chapter, this is due to the maintenance of a proliferative compartment at the retinal periphery known as the ciliary margin zone (CMZ) that is capable of generating all cell types of the retina, thereby acting as a true stem cell compartment (Wetts et al., 1989). In contrast, in “higher” vertebrates such as birds and mammals, this compartment is progressively lost. Some avian (e.g. chick and quail) and marsupial (e.g. opossum) species still have some minimal proliferative and neurogenic activity within a CMZ-equivalent into early postnatal development; however, no evidence of a CMZ has been found in mammals (Fischer and Reh, 2000, Kubota et al., 2002).

Another evolutionary varied aspect of visual development is the presence of binocularity, which has a relatively late emergence in vertebrate evolution. The development of the binocular visual circuit requires the divergence of retinal axons at the optic chiasm such that some project ipsilaterally and some contralaterally, with the degree of binocularity being associated with the size of the ipsilateral retinal projection, and eye position on the head (the more frontal, the more binocular). Many of the molecular mechanisms that guide the ipsilateral projection are evolutionarily conserved, such as the expression of Zic2 and EphB1 homologs in ipsilaterally projecting retinal ganglion cells and the expression of Ephrin-B2 ligand at the optic chiasm midline (Nakagawa et al., 2000, Herrera et al., 2003a, Williams et al., 2003, Lambot et al., 2005). This evolutionarily conserved strategy of conferring ipsilaterality to RGCs raises another question. Do ipsilateral RGCs within species of different vertebrate classes have the same developmental origin? Or is ipsilaterality a new feature in rodents that is purely specified postmitotically?

Through functional characterization of one of these genes, cyclin D2, I found that it is expressed predominantly in a proliferating cell compartment adjacent to differentiating ipsilateral
RGCs during the time window when they are produced. Coincidentally, these cyclin D2-expressing cells are located within the retinal periphery in a zone corresponding to the future location of the CMZ within lower vertebrates. In the null mouse mutant for cyclin D2, the Zic2-expressing ipsilateral RGCs are reduced, particularly during early stages of development, and this reduction is accompanied by a decrease in ipsilaterally projecting axons. Together, my results suggested that a distinct neurogenic zone within the ventral periphery of the retina, much like the postnatal CMZ of lower vertebrates, is defined by cyclin D2 and contributes to the production of a RGC subtype, the ipsilateral RGC.

If future lineage tracing experiments confirm that cyclinD2-expressing cells from the ventral periphery are truly ipsilateral RGC progenitors, then these cells would represent another common strategy used by *Xenopus* and mouse in the generation of ipsilateral RGCs (in addition to Zic2 and EphB receptor expression). As discussed in the previous chapter, *Xenopus* ipsilateral RGCs are produced by proliferating cells of the ventral CMZ (Marsh-Armstrong et al., 1999). These ipsilateral RGC progenitors are specifically induced to proliferate during metamorphosis by thyroid hormone. Thus, in both mouse and *Xenopus*, ipsilateral RGCs may be produced from a specific population of progenitors residing within the ventral periphery of the retina.

Both *Xenopus* and mice, however, have very small ipsilateral projections. In mice, ipsilateral axons only comprise 3-5% of the mature final retinal axon projection. In both species, the ipsilaterally projecting RGCs can be produced from a very small progenitor compartment within the ventral periphery. But is this compartment sufficient in species with greater degrees of binocularity, such as ferrets, cats, and primates where up to 40% of retinal axons are uncrossed? Further experiments need to be carried to dissect out the molecular identity and
behavior of the different proliferative zones within these highly binocular species (Lambot et al., 2005).

The use of a unique progenitor compartment to produce evolutionarily new neuronal subtypes is a strategy that has also been used in other areas of the CNS. *In vivo* fate mapping and *in vitro* clonal analysis identified a glial cell that generates only upper layer neurons in the cortex independently of birthdating (Franco et al., 2012). This fate-restricted radial glial cell goes against the dogma of cortical progenitor cells transitioning through competence states during which they generate neurons that migrate to the different cortical layers (Pearson and Doe, 2004). In primates, upper cortical layers are expanded, allowing for higher level associative connectivity. Thus, addition of this lineage-restricted progenitor pool that specifically produces the upper cortical layers is thought to have facilitated such an evolutionary development (Franco et al., 2012).

**G1 length in the control of neuronal differentiation**

In Chapter 4, I also demonstrated that cyclin D2 has a subtype-specific requirement in retinal development, which raises the question of if and how cyclin D1 and D2 confer their differential influences on retinal cell fate. Quantification of Zic2-expressing RGC and total RGC number in Ccnd2−/− retina showed that while Zic2-expressing RGCs are reduced at E14.5 in VT retina, total RGCs are not. The finding that RGC number remains unchanged leads to several lines of speculation. One possible explanation for this lack of change is due to the decrease in ipsilateral RGCs within the VT retina, negative feedback signals from RGCs are reduced, thus allowing progenitors to increase production of RGCs (not ipsilateral) to compensate for the loss.
A second explanation is that loss of cyclin D2 leads to early depletion of ipsilateral RGC progenitors, and loss of Zic2-expressing RGC production as a consequence.

Both of these possibilities require an exploration of the consequence of cyclin D2 loss on cell cycle progression in driving progenitors. Studies examining changes in cell cycle dynamics resulting from gain or loss of cyclin D function have shown that cyclin D2 overexpression accelerates G1 progression and cyclin D2 loss leads to G1 lengthening (Quelle et al., 1993, Glickstein et al., 2009). G1 lengthening consequently leads to cell cycle exit and differentiation of neural progenitors (Glickstein et al., 2009). In support of this principle, mouse cortical progenitors show G1 lengthening if treated with differentiation factors and G1 shortening if treated with mitogens (Lukaszewicz et al., 2002). Thus, through controlling differentiation decisions, G1 cyclins can control the balance of neurons that are produced at different developmental time points (Caviness et al., 2009). These fate decisions in turn, can be influenced by extrinsic or intrinsic factors that determine the competence state of the progenitor and what cell types it can produce.

One of the peculiar findings of my gene profiling experiments is that ipsilateral RGCs retain the expression of genes expression in progenitor cells postmitotically. The simplest explanation for this finding is that ipsilateral RGCs are less mature than their contralateral counterparts within the retina. However, the additional discovery that cyclin D2 is expressed by proliferating cells that contribute to ipsilateral RGC production offers an alternative, albeit wildly speculative, explanation that cyclin D2 shortens G1 length in the terminal divisions of ipsilateral RGC progenitors as compared to contralateral RGC progenitors. Thus, the differentiation programs normally initiated during this division that downregulate the expression of progenitor genes, such as Sox2 or Math5 (Pevny and Nicolis, 2010, Brzezinski et al., 2012),
may not have enough time to complete their actions, thereby allowing for continued expression of these genes in ipsilateral RGCs. However, further analysis of whether cyclin D2 positive cells in the retina indeed have a shorter cell cycle length than the cyclin D1 only expressing cells of the retina would need to be determined.

**The ventrotemporal retina: a convergence of regulatory mechanisms**

The work that I have presented in this thesis has built upon our current understanding of how the ipsilateral and contralateral RGCs within the retina are molecularly and anatomically distinct. My results have revealed a number of new regulatory mechanisms that may control their production and fate specification, as well as axon pathfinding and connectivity. Importantly, the identification of the proliferating zone of the ventral periphery as a potential progenitor source for ipsilateral RGCs offers an opportunity to integrate some of the molecular pathways we know play a role in the development of the retinal decussations but not how they are interrelated.

In particular, studies on the albino retina have revealed that a disruption in RPE development leads to delayed RGC genesis specifically in the VT retina (Bhansali et al., in preparation), suggesting a particular susceptibility of VT RGC progenitors to whatever RPE signaling pathway is affected the albino retina. However, why a disruption in melanin synthesis, which should affect RPE development throughout the entire retina, has a specific affect on ipsilateral RGC development has been a lingering puzzle. My work suggests that ipsilateral RGCs derive from a progenitor pool with distinct cell cycle regulatory mechanisms, specifically, a dependence on cyclin D2 expression, and thus offers one explanation for how a global signaling defect can lead to a subtype-specific effect.
Concluding remarks

In conclusion, my work on dissecting the molecular identity of ipsilateral and contralateral RGCs and, consequently, the identity of their respective progenitors has uncovered novel mechanisms through which neuronal diversification can be accomplished in the retina. Just as our understanding of the development of the ipsilateral and contralateral retinal pathways have benefited from studies carried out in other developing regions of the CNS as well as in other species, the genes and strategies that I have characterized in the production of specific RGC subtypes can also inform us about what common strategies may be used in diversifying other neuronal subtypes in the retina and elsewhere in the CNS. Already, the D-type cyclins have been shown to have diverse cell-cycle and non-cell cycle dependent functions in regulating fate acquisition. Further study of the molecular mechanisms through which cyclin D2 regulates ipsilateral RGC production will also allow us to further dissect out these molecular strategies.
Chapter 6. Materials and Methods

Animals

C57/BL/6J mice were used as wild-type in this thesis project. Cyclin D2 gene was inactivated by disruption of exons I and II (Sicinski et al., 1996). Cyclin D2^{+/−} mice were provided for breeding by M.E. Ross (Cornell) Holly Moore (NYPI). All experiments Cyclin D2^{−/−} mice were compared to Cyclin D2^{+/+} and ^+/− littermates. Mice were housed in a barrier facility in a timed-pregnancy colony at Columbia University maintained by the Mason lab animal technician, Hranush Melikyan, and exposed only to conditions and procedures that were approved by the Institution Animal Care and Use Committee. Females were checked for vaginal plugs at approximately noon on every weekday. Conception is assumed to take place at approximately midnight, and E0.5 refers to the day on which the vaginal plug was detected.

Anterograde labeling of retinal projections (DiI)

P0 mice were anesthetized intraperitoneally with Avertin (250mg/kg) and perfused transcardially with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (0.1 M PB). Heads of E17.5 embryos or P0 embryos (after perfusion) were post-fixed overnight in 4% PFA in 0.1M PB. Anterograde labeling with DiI was performed from one eye as previously described (Plump et al., 2002). For quantification of the ipsilateral and contralateral projections, pixel intensity of DiI^{+} ipsilateral and contralateral optic tracts adjacent to chiasm midline in a 500x500μm area was measured with MetaMorph image analysis software.
Antibodies

The following primary antibodies were used: rabbit anti-Zic2 (gift of Stephen Brown, 1:10,000), mouse anti-Islet1/2 (gift of Thomas Jessell, 1:50), rabbit anti-Ccnd2 (Santa Cruz, 1:1000), rat-anti Ccnd2 (Santa Cruz, 1:50), mouse anti-Ccnd1 (Santa Cruz, 1:400), goat anti-Brn3 (Santa Cruz, 1:200), mouse anti-Brn3a (Santa Cruz, 1:200), goat anti-Sox2 (Santa Cruz, 1:1000).

The following secondary antibodies were used: Donkey anti-rabbit Alexa488 (Molecular Probes, 1:400), Donkey anti-mouse Alexa488 (Molecular Probes, 1:400), Donkey anti-goat Alexa488 (Molecular Probes, 1:400), Donkey anti-mouse cy3 (Jackson Immunoresearch, 1:500), Donkey anti-rabbit cy3 (Jackson Immunoresearch, 1:500), Donkey anti-goat cy5 (Jackson Immunoresearch, 1:200), Donkey anti-rat Cy3 (Jackson Immunoresearch, 1:500).

Fluorescence-activated cell sorting (FACS)

Dissected retinas were dissociated using papain (Worthington) and resuspended in PBS+2% FBS. FACS was performed at the Columbia University ICRC Core Facility with dead cell exclusion using DAPI to isolate rhodamine-positive live cells.

Generation of ISH probes

Plasmids for making ISH probes were generated for select microarray candidates. cDNA sequence was obtained from NCBI, and PCR primers for generating 500bp riboprobe sequences were selected using Primer-BLAST. 3’ sequences of the gene were targeted if possible. Template cDNA was synthesized from RNA purified from E14-16 retina using the Superscript
III Reverse Transcriptase (Invitrogen), and gene product was generated targeted oligos and Platinum Pfx polymerase (Invitrogen). PCR product was purified using QIAquick PCR Purification kit (Qiagen) and inserted into pCR®-Blunt II-TOPO vector using Zero Blunt Cloning kit (Invitrogen) and transformed into TOP10 or DH5α cells.
Purified plasmids were linearized using restriction enzyme with overnight incubation at 37°C. Riboprobes were synthesized using Digoxygenin RNA labeling mix (Roche). The probes were gifts and so not listed: Math5 (L. Gan, University of Rochester), Sema3e and Sema5d (A. Kolodkin, Johns Hopkins University), Fzd5, Tbx20 (G. Papaioannou, Columbia University).

**Immunostaining of retinal sections**

Embryos were collected at the age of interest in PBS on ice. Heads were decapitated in PBS over ice, fixed in 4% PFA in 0.1M PB for 1hr at 4°C, rinsed in PBS at least 3 times, washed in PBS for a minimum of 1 hour at 4°C, and cryoprotected in 10% sucrose in 0.1M PBS for 1 – 2 nights at 4°C. Heads were embedded in OCT over crushed dry ice and stored at -80°C. 12 µm coronal sections were collected for cyclin D2 immunostaining. 16-20 µm coronal sections were collected for all other stainings. For IHC, slides were blocked in 10%NGS+0.2% triton+PBS for 1 hour, incubated with primary antibody in 1%NGS+0.2% triton+PBS overnight at 4°C, washed 3 x 20 minutes in PBS at room temperature, incubated in secondary antibody in 1%NGS+0.2% triton+PBS overnight at 4°C, and washed in PBS 3 x 20 minutes. Coverslips were mounted on slides with Gelmount.
**In situ hybridization of retinal sections**

Embryos were collected at the age of interest in PBS on ice. Heads were decapitated in PBS over ice, fixed in 4% PFA in 0.1M PB at 4°C overnight, rinsed in PBS at least 3 times for a minimum of 1 hour total at 4°C, and cryoprotected in 30% sucrose in 0.1M PBS for 1 – 2 nights at 4°C. 14 μm coronal sections were collected through the retina and immediately processed for *in situ* hybridization (or stored at -80°C) using a protocol adapted from Kuwajima *et al.* (Kuwajima *et al.*, 2012). After ISH hybridization, tissues were fixed for 30 minutes in 4% PFA, washed with PBS, then processed for immunostaining with Zic2, Isl1/2 and Islet1/2 using normal IHC methods.

**Imaging**

ISH and wholemount preparations (antero- and retrograde labeling) were taken by Axiovision software through the Axiophot camera connected to a Zeiss Axioplan2 microscope. IHC preparations were imaged using a Zeiss AxioImager M2 microscope equipped with Apotome, AxioCam MRm camera, and Neurolucida software (V10.40, MicroBrightField Systems). The Apotome provides a resolution that is comparable to that provided by a confocal microscope.

**Microarray preparation and analysis**

Amplified cDNA was generated from purified RNA using Ovation Pico WTA System (Nugen) and then labeled and fragmented using the Encore Biotin Module (Nugen). Labeled cDNA was hybridized on Mouse Genome 430 2.0 Array chips (Affymetrix), and analyzed using
GeneSpring GX11 (Agilent). Differentially expressed genes were identified from 3 biological replicates (3 independent rounds of retrograde labeling and FACS) by greater than two fold change and corrected \( p < 0.05 \), Benjamini-Hochberg.

**Quantitative RT-PCR (Table 3, oligos)**

cDNA was retrotranscribed from purified RNA using the Superscript III Reverse Transcriptase (Invitrogen). qPCR was performed using the Stratagene MX3000 machine with the Sybr Green PCR Kit (Applied Biosystems). Transcript levels were normalized to that of HPRT. qPCR-specific primers are designed using the Primer3 program.

**Quantification of RGCs in retinal sections**

Every third section of a 16 \( \mu \text{m} \) series was used for analysis. All quantification for sections processed for IHC was performed using Meta Imaging Series Metamorph 7.0. The inner surface of the retina was divided into radial sectors of equal width (50 \( \mu \text{m} \)), starting at the most peripheral Isl1/2 or Zic2 expressing cell, and divided by a line drawn from the inner surface of the retina towards the outer surface in the direction of the migrating cells. Cell numbers were recorded for each individual sector. Cumulative Zic2 counts from all sections of the series were totaled for final value. Images were thresholded in the Metamorph system such that only cells brighter than Zic2\(^+\) progenitor cells within the apical progenitor layers were not counted. Retinal area in sections was measured using the Region Tracing Tool on the Metamorph imaging system. The section including and 3 section caudal to the optic nerve were used for Isl1/2 statistical analysis at E14.5. These sections were chosen because the decrease in Zic2\(^+\) RGCs was greatest within these sections in \( \text{Ccnd2}^{-/-} \) animals.
**Retrograde labeling**

Dyes used were labeling of axon projections were: tetramethylrhodamine-conjugated dextra MW3000, anionic, lysine fixable (Molecular Probes, D-3308); fluorescein-conjugated dextra MW3000, anionic, lysine fixable (Molecular Probes, D-3306); Cholera Toxin Subunit B (Recombinant), Alexa Fluor® 594 Conjugate (C-22842).

Pregnant females were anesthetized using either Avertin (250mg/kg) or ketamine–xylazine (100 and 10 mg/kg, respectively, in 0.9% saline). While the mother was kept alive, each E16.5 embryo was removed and decapitated in DMEM/F12 buffer over ice. The head was immersed in DMEM and hard palate was removed to expose the optic chiasm and tract. The optic tract and surrounding tissue was dried and a pinch of dextran was applied to the severed optic tract with forceps. For RD3000: the head was incubated in pre-oxygenated bubbling ACSF for approximately 2-3 hours. Each retina was dissected out of the head and processed for FACS. For FD3000: the head was incubated in pre-oxygenated bubbling ACSF for approximately 2-3 hours and then transferred to 4ºC overnight for further incubation.

For whole-mount imaging preparations, labeled heads were fixed in 4% PFA in 0.1M PB at 4ºC overnight and rinsed in PBS. Retinas were dissected and flat-mounted on coverslipped slides with Gelmount.

**RNA extraction**

RNA purification was performed Absolutely RNA Nanoprep kit (Stratagene) or RNeasy kit (Qiagen). RNA concentration was determined by Nanodrop spectrophotometer or RNA 6000 Pico kit (Agilent) using Agilent 2100 Bioanalyzer and then stored at -80º C.
**Statistical Analysis**

All were evaluated for statistical significance using student’s two-tailed unpaired t-test in Microsoft Excel. All error bars represent standard error of the mean (SEM). p-values are indicated as follows: *p < 0.05; **p<0.01; ***p < 0.001.
References


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