Progenitor Cell Maturation and Initiation of Neurogenesis in the Developing Vertebrate Neural Retina

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Abstract

The mature vertebrate central nervous system is composed of an enormous number of neuronal and glial cells. A relatively small number of progenitor cells generate these cells during a finite period of time of development. Progenitor cells during early stages of central nervous system development divide so that each division produces two progeny that divide again. This ‘preneurogenic’ mode of division is essential for the exponential increase of number of progenitor cells. Later, progenitor cells change their mode of division to ‘neurogenic’, where one or both daughter cells produced by a division withdraw from the mitotic cycle and differentiate. This more mature, neurogenic division is critical for generation of a functional nervous system. The aim of the project described in this thesis was to understand: 1) the molecular differences that dictate the two modes of progenitor cell division, namely preneurogenic and neurogenic, 2) the mechanism that regulates the switch in the mode of division, and 3) the molecular trigger that initiates differentiation.

Molecular differences between preneurogenic and neurogenic progenitor cells were identified, and are described in more detail in chapter II. The early, preneurogenic progenitor cells express the transcription factor, Sox2, and a ligand for the Notch receptor, Delta1. The more mature, neurogenic progenitor cells express Sox2 and the bHLH transcription factor, E2A, and do not express Delta1. Perturbation of Notch signaling resulted in conversion of progenitor cells from preneurogenic to neurogenic and in premature neurogenesis. Furthermore, Sonic hedgehog was found to be expressed by a subset of newly differentiating cells. Misexpression of Sonic hedgehog led to premature maturation of preneurogenic progenitor cells and neurogenesis. These results suggest that Notch signaling maintains progenitor cells in the preneurogenic state and that Sonic hedgehog

initiates progenitor cell maturation.

Certain proneural bHLH transcription factors were found to initiate neurogenesis, and are described in more detail in chapter III. Expression of a number of proneural bHLH factors comes up in a stereotypic temporal sequence prior to the onset of ganglion cell differentiation. Ascl1 and Neurog2 were expressed first, which was followed by expression of Neurod1 and Neurod4. Finally, Atoh7 was expressed, which preceded the appearance of ganglion cells. Individual progenitor cells expressed heterogeneous combinations of proneural genes prior to ganglion cell genesis. Misexpression of Ascl1 or Neurog2 in preneurogenic retina was sufficient to initiate ganglion cell genesis. Misexpression of Neurog2 initiated the stereotypic sequence of proneural gene expression that normally preceded ganglion cell genesis. Ascl1 expression was also sufficient to initiate ganglion cell genesis. However, it functioned by a mechanism distinct from that of Neurog2. These results suggest that ganglion cell genesis may be initiated by two different mechanisms.

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CHAPTER I.

INTRODUCTION

I.A. Overview of the Thesis

The mature central nervous system of the vertebrate species such as human is composed of thousands of neuronal and glial cell types with the total cell count reaching over 100 billion (Williams and Herrup, 1988; Pakkenberg and Gundersen, 1997). These enormous numbers of cells are generated from a relatively small number of progenitor cells, the dividing cells of the developing nervous system. Initially in the developing central nervous system, the number of progenitor cells expands exponentially without generating postmitotic cells. This is through a series of 'preneurogenic' cell divisions, in which both progeny of each division divide again. Later in development, progenitor cells switch their mode of division to a 'neurogenic' mode. In this mode of division, one or both progeny of a division withdraw from the mitotic cycle and differentiate as neurons or glia. This later neurogenic mode of progenitor cell division is essential for generation of the functional nervous system. Despite considerable progress having made in understanding important developmental processes, the following fundamental questions remain unanswered: 1) What molecular differences dictate the differential modes of progenitor cell division, namely preneurogenic and neurogenic, 2) what is the mechanism that regulates the switch in the mode of division, and 3) what factor is responsible for initiation of differentiation? This thesis addresses these questions using the embryonic chick retina as a model. Chapter I provides background and rationale of this thesis. Chapter II describes the molecular differences between preneurogenic and neurogenic progenitor cells and the mechanism underlying progenitor cell maturation. Chapter III investigates the role of proneural bHLH genes in initiating neurogenesis in the retina.

I.B. Embryology of the Vertebrate Retina

The vertebrate neural retina is a region of central nervous system, which lines the inner surface of the back of the eye. It contains photoreceptor cells that sense the light coming through the lens and convert it into the neuro-electrical impulses. The converted signal is transmitted through the interneurons of the retina and eventually to ganglion cells. Ganglion cells send the information to various visual centers of the brain for further processing.

The vertebrate neural retina originates developmentally from the anterior region of the neural tube (Zuber and Harris, 2006). The region specified to become retina, known as the eye field, is initially a single field and later separates into two eye primordia, one on each side of the forebrain. Subsequently, each of the eye primordia evaginates out of the diencephalon to form an optic vesicle. The optic vesicle invaginates to form a two-layered optic cup, and the inner layer develops into the neural retina in response to an inductive signal from the overlying head ectoderm. The outer layer of the optic cup is a single layer of epithelial cells, and it eventually becomes the retinal pigmented epithelium. The optic cup remains attached to the brain through the hollow optic stalk. Retinal ganglion cells grow long projection axons through the inferior wall of the optic stalk, which later becomes the optic nerve. While the optic cup is forming, other structures of the eye are also induced. The contact between optic vesicle and the overlying ectoderm induces the formation of the lens placode, which invaginates to develop into the lens and in turn induce cornea from the overlying ectoderm. The optic cup also contributes cells to some of the peripheral structures of eye. Cells located at the peripheral rim of the optic cup contribute to the iris and ciliary body. The remaining eye

structure including sclera and extra ocular muscles are developed from neural crest cells and head mesoderm.

Retinal progenitor cells undergo multiple rounds of cell division and ultimately generate six neuronal and one glial cell type. Initially, retinal progenitor cells at the optic vesicle and early optic cup stages divide so that each division produces two cells that divide again. This mode of cell division results in an exponential increase in the number of retinal progenitor cells. Therefore, this early mode of progenitor cell division is essential for the rapid growth of the tissue within the limited time of normal development. Hereafter, progenitor cell divisions producing two progenitor cells without producing any postmitotic cells will be designated as 'preneurogenic' division. Later, progenitor cells change their mode of division and begin to generate postmitotic neurons. Once the switch of cell division modes has occurred, progenitor cells undergo asymmetric divisions in which one of the daughter cells produced by a division withdraws from the mitotic cycle and differentiates whereas the other daughter cell remains as a progenitor cell. Following a few rounds of asymmetric division, progenitor cells undergo terminal division where both daughter cells begin to differentiate. These later modes of progenitor cell division, in which one or both progeny become postmitotic, will be designated as 'neurogenic' division. This later, neurogenic mode of divisions is critical for generation of a functional nervous system.

Progenitor cell maturation, that is the switch in the cell division mode of progenitor cells, first occurs in a small number of progenitor cells located in the center of the retina (Prada et al., 1991; Reese and Colello 1992; McCabe et al., 1999). It will form a 'neurogenic zone' in the center with neurogenic progenitor cells and their postmitotic progeny. A sharp boundary or the 'neurogenic front' can be marked by the most peripherally located postmitotic cells and separates the neurogenic zone in the center

from the preneurogenic zone in the periphery. Progenitor cells located peripheral to the neurogenic front undergo preneurogenic divisions (Dutting et al., 1983). The preneurogenic progenitor cells just peripheral to the neurogenic front soon switch their mode of division to neurogenic, and thereby the neurogenic front advances peripherally. The advance of the neurogenic front continues until the neurogenic front reaches the peripheral margin of the optic cup and the entire neural retina become neurogenic. In fish and amphibia, however, dividing cells are retained in the periphery of the mature retina, the ciliary marginal zone (CMZ). These retinal progenitor cells continuously produce new neurons in the retina throughout the life of an animal (Straznicky and Gaze, 1971; Johns, 1977; Wetts et al., 1989).

Once neurogenesis commences in the developing neural retina, a seemingly homogenous pool of retinal progenitor cells gives rise to six types of retinal neurons and one type of glial cell (Turner and Cepko, 1987; Holt et al., 1988; Wetts et al., 1989; Turner et al., 1990; Fekete et al., 1994). These seven major cell types of the retina are generated in a stereotypic sequence, which is conserved across vertebrate species (Carter-Dawson and LaVail, 1979; Young, 1985; La Vail et al., 1991; Stiemke and Hollyfield, 1995; Cepko et al., 1996; Hu and Easter, 1999): Retinal ganglion cells are generated first and are followed by generation of amacrine cells, horizontal cells, and cone photoreceptor cells. Rod photoreceptor cells, bipolar cells, and Müller glial cells are produced later in the development. The time during which each of the retinal cell types is generated, however, exhibits considerable overlap (Young, 1985; Stiemke and Hollyfield, 1995).

Similar to the other regions of the brain, the neural retina forms a highly laminated structure. The retinal cell types are organized into layers. Three layers of cell bodies are separated by two layers of axons and dendrites. Retinal progenitor cells

undergo mitosis near the ventricular surface (i.e. towards pigmented epithelium), and the postmitotic cells migrate to the proper layer of the retina. Ganglion cells, the first neurons generated in the retina, migrate to the innermost layer of the retina (i.e. towards vitreous) and form the ganglion cell layer (GCL). Just outside of the GCL is the inner nuclear layer (INL). Interneurons of the retina, both excitatory and inhibitory, migrate to and differentiate in this layer. These interneurons include horizontal cells, bipolar cells, and amacrine cells. A subset of amacrine cells, the displaced amacrine cells, resides in the GCL. Cone and rod photoreceptors form the outer nuclear layer (ONL), the outermost cell layer. Interposed between these three cell layers are the inner and outer plexiform layers (IPL and OPL). These layers are composed of processes of the retinal cells, and synaptic connections between retinal neurons are formed in these layers. Muller glia span the entire thickness of the retina. This architecture allows serial processing of visual information. Light is first detected by photoreceptors. Photoreceptor cells convert light stimulation into nerve impulses, which is transmitted through interneurons to the ganglion cells. Ganglion cells have long projecting axons that send the signal to the brain, where the information is processed and interpreted as visual perception.

The vertebrate neural retina is a region of the central nervous system with easy accessibility and a relatively simple structure. Understanding of many fundamental developmental events comes from studies of the neural retina as a model. The embryonic neural retina is also a very suitable system to study the mechanism for progenitor cell maturation and initiation of neurogenesis. As described above, neurogenesis of the developing neural retina is first apparent in the center and expands towards the periphery as development progresses. Thus, for a period of development, a cross section of the embryonic retina contains an array of cells with various maturation stages along the central-to-peripheral axis. Studies described in this thesis use the

developing retina to study the mechanisms underlying progenitor cell maturation and initiation of neurogenesis.

I.C. Molecular Differences

between Preneurogenic and Neurogenic Progenitor Cells

I.C.i. Issue

The entire neural retina is derived from a relatively small number of progenitor cells. All cells of the optic vesicle and the early optic cup are dividing progenitor cells, and no postmitotic cells are present. These cells undergo preneurogenic division in which both resulting daughter cells divide again. This series of preneurogenic divisions generates a large pool of progenitor cells, which is essential for growth of the tissue during the finite period of normal retinal development. Later in development, retinal progenitor cells switch their mode of division so that one or both daughter cells exit the mitotic cycle and begin to differentiate. Several rounds of neurogenic division of progenitor cells generate an array of retinal neurons and glial cells, which is essential for generation of the functional neural retina. A fundamental question that remains to be answered is what are the molecular differences between preneurogenic and neurogenic progenitor cells?

I.C.ii. Current Model from Studies in Other Tissues of the Central Nervous System

The nature of preneurogenic and neurogenic progenitor cell divisions in the retina is poorly understood. Most studies addressing the molecular differences between the two progenitor cell stages focused on other regions of the developing vertebrate central nervous system including the developing cerebral cortex. As retinal progenitor cells have the same origin as progenitor cells of other tissues of the central nervous system, it is likely that preneurogenic and neurogenic progenitor cells of other parts of the central

nervous system share common molecular characteristics with their counterparts in the retina.

Orientation of Cell Cleavage and Differential Distribution of Numb

The current model suggests that the orientation of the cell cleavage plain during progenitor cell division and the differential distribution of the Numb protein to the two daughter cells can influence the mode of progenitor cell division in the developing cortex. Studies suggested that progenitor cells with a cleavage plane perpendicular to the ventricular surface divide symmetrically generating daughter cells that divide again, whereas progenitor cells with a cleavage plane parallel to the ventricular surface divide asymmetrically generating one daughter cell close to the ventricular surface that divides again and the other daughter cell away from the ventricular surface differentiates (Martin, 1967; Chenn and McConnell, 1995). The unequal inheritance of fate-determining molecule such as Numb has also been implicated in influencing the mode of progenitor cell division (Wakamatsu et al., 1999).

These findings led to the investigation of the role of cell cleavage plane during progenitor cell division and the distribution of Numb protein to the resulting daughter cells in the division mode of retinal progenitor cells. In contrast to the findings in the developing cortex, however, neither the plane of cell cleavage nor the distribution of the Numb protein correlated to the mode of progenitor cell division in the developing retina (Silva et al., 2002). No difference was found in the frequency of the orientation of the cleavage planes between preneurogenic or neurogenic zone of the developing retina. Furthermore, the Numb protein was present both in the dividing and differentiating daughter cells unlike what has been suggested in the developing cortex.

Tis21 and Mnb Expression by Neurogenic Progenitor Cells in Cortex

Several classes of molecules have been linked to the neurogenic division of progenitor cells in other regions of central nervous system. Tis21 (also known as Btg2 or PC3) is one such example. Initially, it was described as an immediate early gene whose expression was induced during neuronal differentiation by growth factors and tumor promoters in PC12 cells and Swiss 3T3 cells, respectively (Lim et al., 1987; Bradbury et al., 1991; Fletcher et al., 1991; Rouault et al., 1996). Tis21 mRNA, which is expressed transiently during G1 phase of the cell cycle, was shown to label the neurogenic progenitor cells in the neural tube (Iacopetti et al., 1994; Iacopetti et al., 1999; Haubensak et al., 2004). The Tis21 protein, on the other hand, persists through mitosis and is present in postmitotic cells (Iacopetti et al., 1999).

Mnb (also known as Dyrk1A) also has been linked to the neurogenic mode of division. Characterization of Mnb expression in the developing chick neural tube predicted that expression of Mnb precedes the switch of the mode of division from preneurogenic to neurogenic and that asymmetric inheritance of Mnb to one of the daughter cells produced by preneurogenic division may lead to the switch of cell division mode to neurogenic (Haubensak et al., 2004).

Previous characterization of Tis21 and Mnb expression raises the possibility that they may be a common molecular marker for neurogenic progenitor cells in various tissues of the central nervous system. Expression of both Tis21 (Haubensak et al., 2004) and Mnb (Song et al., 1996) was found in the developing mouse retina. Since preneurogenic progenitor cells are maintained only for a short period of development in mouse retina, their expression was not characterized in relation to the mode of progenitor cell division. Thus, it will be worth testing whether their expression is specific to retinal progenitor cells undergoing neurogenic division. Due to the limited availability

of the reagent, expression of Tis21 and Mnb in retinal progenitor cells was not investigated in this thesis.

E Proteins and Components of Delta-Notch Signaling

E proteins and components of Delta-Notch signaling were also considered as candidate molecules whose expression can distinguish preneurogenic and neurogenic progenitor cells from each other. E proteins are binding partners of proneural bHLH transcription factors (Lee et al., 1995; Naya et al., 1995 Gradwohl et al., 1996). Although expression of E proteins is thought to be ubiquitous (Roberts et al., 1993), the role of E proteins has been implicated in several aspects of neuronal differentiation (reviewed in Ik Tsen Heng and Tan, 2003). This raises the possibility that expression of E protein in the central nervous system may be specific to neurogenic progenitor cells. On the other hand, components of Delta-Notch signaling may mark preneurogenic progenitor cells. Delta-Notch signaling has been linked to inhibition of neurogenesis (Dorsky et al., 1995, 1997; Henrique et al., 1997; Scheer et al., 2001; Jadhav et al., 2006). In addition, cells in the caudal stem zone of the developing neural tube, which is analogous to the preneurogenic progenitor cells, are characterized with the uniform expression of Delta, the ligand for Notch (Henrique et al., 1995; Caprioli et al., 2002; Akai et al., 2005).

E proteins and Delta-Notch signaling will be discussed further in the following section (I.C.iii).

I.C.iii. E Proteins, Class I HLH Family Transcription Factors

Introduction to E Proteins

E proteins include E2A, which encodes two splice variants E12 and E47, (Murre et al., 1989), HEB, which encodes two splice variants ME1a and ME1b, (Hu et al., 1992), and

E2-2 (Henthorn et al., 1990; Soosaar et al., 1994). Together, these proteins compose the class I HLH family, a subclass of the helix-loop-helix (HLH) transcription factor family. They share highly conserved sequences such as a basic domain and a HLH domain. The HLH domain, common to all HLH family transcription factors, is composed of two amphipathic helices interrupted by a loop (Murre et al., 1989). It is responsible for formation of a homo- or heterodimer, the functional unit for transcription regulation. E proteins also contain a conserved basic domain. The basic domain allows binding to specific DNA sequences, referred to as a hexanucleotide E-box sequence (Murre et al., 1989).

Function of E Proteins

E proteins are expressed widely in most embryonic and adult tissues (Roberts et al., 1993). In nervous system, the expression of E2A, an E protein family member, is found in the proliferative layer of the neuroepithelium throughout embryonic stages (Roberts e al., 1993). In addition, it is also found in the subventricular zone of the mature nervous system, a region of countinuous neurogenesis (Roberts et al., 1993). This broad expression of E2A in neural progenitor cells suggests an important role of E2A in neuronal development. Expression of other members of E protein family such as HEB and E2-2 is also found in regions of the nervous system with ongoing progenitor cell division (Soosaar et al., 1994; Chiaramello et al., 1995).

Despite the ubiquitous expression in the nervous system throughout the period of neurogenesis, the function of E proteins is largely unknown. Targeted deletion of E2A did not result in an apparent defect in neurogenesis (Zhuang et al., 1992, 1996; Bain et al., 1994). Likewise, overexpression of E2A in uncommitted P19 cells failed to stimulate neuronal differentiation in contrast to overexpression of Ascl1, a class II HLH protein

(Farah et al., 2000). Nevertheless, studies of daughterless, the class I HLH gene in Drosophila, provided an important insight into the role of E proteins. Loss of daughterless resulted in severe defects in neuronal precursor differentiation (Vaessin et al., 1994; Hassan and Vaessin 1997). These studies suggest that daughterless plays a key role in neurogenesis. It is important to note that daughterless is the only member of E protein family in Drosophila. The lack of an obvious phenotype in E2A loss of function studies in vertebrates suggests functional redundancy among E protein family members. Significantly, experiments showed that the function of E2A can be replaced with HEB during B cell development (Zhuang et al., 1998). Furthermore, all three E proteins have a comparable ability to promote neuronal differentiation when introduced into P19 cells together with Neurod2 (Ravanpay and Olson, 2008). Collectively, these observations suggest that E proteins in the vertebrate species have redundant function and that the importance of E protein function in the vertebrate nervous system remains to be determined.

Consistent with the function of the Drosophila counterpart, studies in the nervous system and other tissues suggests the role of E proteins in the neuronal differentiation. First, E proteins are binding partners of proneural bHLH transcription factors. In the nervous system, proneural genes, members of the class II HLH family, play a key role in neuronal differentiation and cell fate specification (reviewed in Bertrand et al., 2002). Although expression of specific proneural genes is restricted to certain neuronal lineages, they form dimers with ubiquitously present E proteins. Significantly, the dimerization between proneural bHLH proteins and E proteins is important in forming a functional transcription activator. Homodimers of class II proteins have only limited transcription activity, and dimerization with E proteins is essential for efficient function of proneural genes (Akazawa et al., 1992; Johnson et al., 1992; Shimizu et al., 1995; Gradwohl et al.,

1996; Peyton et al., 1996). Second, E2A can negatively regulate the cell cycle independent of class II HLH transcription factors. Although its function in suppression of cell cycle was not directly tested in the context of neuronal differentiation, the results from in vitro studies are consistent with its role in promoting neuronal differentiation. Analysis of E2A expression in synchronized fibroblasts showed that E2A expression level is regulated according to the proliferative status of the cell with its expression being high during cell cycle arrest and low during re-entry to the cell cycle (Loveys et al., 1996). In addition, transient transfection of E2A in NIH3T3 cells showed that E2A suppresses cell division presumably by direct transcriptional activation of cyclin-dependent kinase inhibitors such as p21, p15, and p16 (Peverali et al., 1994; Prabhu et al., 1997; Pagliuca et al., 2000). Collectively, these studies raise the possibility that E proteins may promote neurogenesis by suppressing cell cycle progression and later by binding to proneural bHLH transcription factors and activating the neurogenic machinery.

E Proteins as a Putative Molecular Marker of Neurogenic Progenitor Cells

It is believed that expression of E proteins is ubiquitous throughout development. The proposed function of E proteins in the neuronal differentiation, however, raises the possibility that they are active only as neurogenesis commences. Initially, progenitor cells of the developing central nervous system divide without neuronal differentiation. Previous observations of E proteins have implicated them in neuronal differentiation. Therefore, it seems likely that expression of E proteins comes up as progenitor cells switch their mode of division to neurogenic and begin to generate postmitotic neurons.

Consistent with this notion, northern blot analysis of mouse brain at various embryonic ages suggests that the expression of murine E proteins is initiated as early as E7 (Ravanpay and Olson, 2008). This stage coincides with the time of the first

appearance of postmitotic neurons in the developing mouse brain. Therefore, I propose that E proteins are expressed specifically by progenitor cells that are generating neurons and not by preneurogenic progenitor cells. .

I.C.iv. Delta-Notch Signaling

Introduction to Delta-Notch Signaling

Notch signaling (Reviewed in Artavanis-Tsakonas et al., 1999), regulates a large array of cellular processes including differentiation, proliferation, and apoptotic programs in invertebrates and vertebrates. In the developing nervous system including retina, Notch signaling is known to negatively regulate neurogenesis. Thereby, it maintains progenitor cells and later regulates the switch from neurogenesis to gliogenesis (Austin et al., 1995; Dorsky et al., 1995; Bao and Cepko, 1997; Dorsky et al., 1997; Henrique et al., 1997; Furukawa et al., 2000; Gaiano et al., 2000; Scheer et al., 2001). Based on these known functions of Notch signaling in the developing retina, it is possible that Notch signaling is correlated with the preneurogenic mode of progenitor cell division.

The Notch gene, first characterized in Drosophila, encodes a 300kD single spanning transmembrane receptor. The large extracellular domain contains 36 tandem epideral growth factor (EGF)-like repeats and three membrane proximal, cysteine-rich Lin12/Notch/Glp-1 (LNG) repeats. The RAM domain, six ankyrin repeats, a transcriptional activator domain (TAD), and a proline-, glutamine-, serine-, threonine-rich (PEST) sequence are found in the intracellular domain (Wharton et al., 1985; Kidd et al., 1986). Notch activation involves proteolytic cleavages at three sites, S1, S2, and S3. S1 cleavage occurs within the secretory pathway so that a processed heterodimeric form is transported to the cell surface. S2 and S3 cleavages occur following ligand binding. Four

members of Notch receptors, Notch1 to Notch4 are expressed in vertebrates. Ligands for Notch signaling include Delta and Jagged, which are also transmembrane proteins (Reviewed in Artavanis-Tsakonas et al., 1999). Other important components of the pathway include DNA binding protein CSL (CBF-1 or C promoter binding factor-1, Su(H) or Suppressor of Hairless, or LAG-1) (Reviewed in Artavanis-Tsakonas et al., 1999).

Activation of Notch signaling requires direct cell-cell interaction as both the ligand and receptor are transmembrane proteins (reviewed in Artavanis-Tsakonas et al., 1999). Upon activation, the Notch receptor is cleaved, initially at the S2 site by TACE (TNF-α converting enzyme) and at the S3 site by the γ-secretase complex, which includes the transmembrane proteins presenilin and nicastrin. S2 cleavage releases a membrane tethered form of the Notch intracellular domain (NICD). The subsequent S3 cleavage releases the soluble intracellular domain of Notch. NICD binds to CSL, and together they interact with a transcriptional activation complex including Mastermind, the histone acetyltransferase p300, and PCAF (p300/CBP-associated factor). This transcription activation complex induces expression of members of Hes and HRP/HERP/Hey families. These genes encode transcriptional repressors that repress expression of proneural bHLH transcription factors, whose expression would result in neurogenesis. In the absence of NICD, CSL represses transcription of Hes and HRP/HERP/Hey genes through interactions with a co-repressor complex containing HDAC.

Function of Notch Signaling in the Developing Vertebrate Retina

The ligand Delta and the receptor Notch are expressed in the developing retina of various species (Ahmad et al., 1995, 1997; Austin et al., 1995; Lindsell et al., 1996; Bao and Cepko 1997; Dorsky et al., 1997; Henrique et al., 1997). Delta1 and Delta4, ligands for Notch, are expressed in scattered isolated cells in the developing retina both in chick

and mouse (Ahmad et al., 1997; Henrique et al., 1997; Nelson and Reh, 2008, 2009). The cells positive for Delta1 and Delta4 are newly differentiating postmitotic cells (Henrique et al., 1997; Nelson and Reh, 2008). Notch, the receptor of the pathway, is uniformly expressed by retinal progenitor cells (Austin et al., 1995; Ahmad et al., 1997). Notch signaling is known to negatively regulate neuronal differentiation, and thereby maintain the pool of retinal progenitor cells. Constitutive activation of Notch in the early developing retina blocked differentiation and caused all cells to continue to divide (Dorsky et al., 1995, 1997; Henrique et al., 1997; Scheer et al., 2001). Conversely, blocking Notch activity resulted in premature differentiation and reduced cell proliferation (Jadhav et al., 2006; Yaron et al., 2006; Nelson et al., 2007). The inhibition of neuronal differentiation by Notch signaling takes place largely by the repression of proneural gene expression via Hes1 and Hes5 (Nishimura et al., 1998; Kageyama et al., 2007). Notch signaling is also implicated in the cell fate specification in the retina, which will not be discussed in this thesis.

Components of Notch Signaling as a Putative Molecular Marker of Preneurogenic Progenitor Cells

The known function of Notch signaling in inhibition of neurogenesis suggests the possibilities that Notch signaling plays a role in maintenance of preneurogenic progenitor cells and that expression of key players of Notch signaling is specific to preneurogenic progenitor cells. However, expression of components of Notch signaling in the preneurogenic retina was not characterized. Studies from caudal stem zone of the neural tube support the possibility that components of Notch signaling may be specific molecular markers of preneurogenic progenitor cells. Analogous to preneurogenic progenitor cells in the retina, cells located in the caudal stem zone continuously divide

without generation of differentiating cells and add new progenitor cells to the developing neural tube (Mathis et al., 2001). Studies report the uniform expression of components of Notch signaling including Delta1 and Notch1 in caudal stem zone (Henrique et al., 1995; Caprioli et al., 2002; Akai et al., 2005). Thus, it is worth testing whether the similar uniform expression of Delta1 also marks the preneurogenic progenitor cells in the early developing retina.

I.C.v. Hypothesis

A fundamental question of how preneurogenic and neurogenic progenitor cells are molecularly distinct from each other was addressed in this thesis. Several factors that distinguish the two stages of progenitor cells have been suggested based on work in other tissues of the developing vertebrate central nervous system. However, some of the proposed factors including the orientation of cell cleavage plane and the differential inheritance of Numb protein failed to explain the differences between the two stages of progenitor cells in the retina as discussed above. Other molecular components such as E proteins and the components of Delta-Notch signaling were considered instead. Although their expression was not directly assessed relative to preneurogenic and neurogenic progenitor cells stages, previous observation raised the possible correlation. Thus, it was hypothesized that components of Delta-Notch signaling such as Delta1 can specifically identify the preneurogenic progenitor cells in the developing retina whereas expression of E proteins can identify the neurogenic progenitor cells. This hypothesis was tested in this thesis, and the results are presented in chapter II.

I.D. Regulation of Progenitor Cell Maturation

I.D.i. Issue

A large number of cells that comprise the mature central nervous system are generated from a relatively small number of progenitor cells. The initial preneurogenic mode of division accounts for the rapid increase in progenitor cell number during the limited period of time of the normal development. As development progresses, progenitor cells switch their mode of division from preneurogenic to neurogenic, in which one or both daughter cells produced by a division withdraw from the cell cycle and begin to differentiate. There is only limited increase in the number of cells with neurogenic mode of division. Rather, this later, neurogenic mode of progenitor cell division is critical for generating functional neurons and glia. The underlying mechanisms for maintenance of the preneurogenic mode of cell division and the later transition of the mode from preneurogenic to neurogenic are poorly understood.

I.D.ii. Candidate Factors for Regulator of Progenitor Cell Maturation

Generation of postmitotic, differentiating neurons in the retina is a readily identifiable sign of the progenitor cell maturation. In the retina, the generation of postmitotic cells begins in the center of the retina and progressively expands to its peripheral margin. Multiple factors have been implicated in this process in the retina. Secreted signaling molecules such as Hedgehog (Hh) and Fibroblast Growth Factor (FGF) have been shown to positively regulate the central-to-peripheral expansion of the region with neurogenesis, whereas Wnt, another secreted signaling molecule, appears to maintain progenitor cell proliferation in the peripheral region of the retina. In addition, observations in other tissues of the developing central nervous system implicated a cell intrinsic timer

mechanism and Notch signaling in positive and negative regulation of the onset of generation of postmitotic cells, respectively. In the following sections, relevant studies implicating each of these factors in controlling the onset of postmitotic neuron production will be further examined.

I-D-iii. Intrinsic Timer Mechanism

Introduction to the Intrinsic Timer Mechanism

Several studies have demonstrated an intrinsic timer program that operates in cells of developing tissues including the central nervous system. This mechanism regulates the sequence of certain developmental events. These findings are largely based on studies of the behavior of oligodendrocyte precursor cells (OPCs) in culture. When individual cells were isolated from embryonic rat brain or early postnatal rat optic nerve and cultured in vitro, they were able to correctly determine when to stop dividing and to begin differentiation (Abney et al., 1981; Raff et al., 1985; Temple and Raff, 1986). Furthermore, the isolated OPCs generated different glial cell types in the same time schedule as they would have in vivo (Abney et al., 1981; Raff et al., 1985; Temple and Raff, 1986).

It has been suggested that cells can count the number of cell divisions. The progeny of an individual OPC isolated from rat optic nerve underwent the same number of divisions for about the same period of time before they stopped dividing and differentiated (Temple and Raff, 1986). Some proteins have been identified as components of the intracellular timer. It turned out that the progressive increase in the level of p27 and p18, cyclin dependent protein kinase (Cdk) inhibitor and the gradual decrease in the level of Id4, an inhibitor of bHLH transcription factor family proteins,

account, at least in part, for the intrinsic timer mechanism (de Nooij et al., 1996; Fero et al., 1996; Kiyokawa et al., 1996; Lane et al., 1996; Nakayama et al., 1996; Durand et al., 1997, 1998; Franklin et al., 1998; Hong et al., 1998; Kondo and Raff, 2000; Tokumoto et al., 2002; Marin-Husstege et al., 2006). Each of these proteins appears to account only for a part of the molecular nature of the intrinsic timer. For instance, loss of function or gain of function experiments for p27 only delayed or accelerated the timer, respectively, without a complete impairment (Durand et al., 1998; Tokumoto et al., 2002). This suggests that additional components participate in the cell intrinsic timer mechanism in addition to previously identified molecules such as p27, p18, and Id4. Thus, despite some progress, the molecular nature of the cell intrinsic timer mechanism still remains to be elucidated. Further work will be necessary to completely understand the molecular nature of the cell intrinsic timer mechanism.

Intrinsic Timer Mechanism in the Vertebrate Neural Retina

There is some evidence for a cell intrinsic timer mechanism in the developing retina. Studies from a heterochronic culture system (i.e. a culture system in which cells from different developmental ages are mixed and cultured together) showed that an intrinsic timer mechanism exists in retinal cells. Cells isolated from E15 rat retina began to produce rod photoreceptors only after five days in a pellet culture as they would normally do in situ. Significantly, they also produced rod cells on schedule when cultured together with a 50-fold excess of retinal cells from an older retina, which generate mainly rod photoreceptors at the beginning of the culture period (Watanabe and Raff, 1990). This, along with other findings (Cayouette et al., 2003) suggests that an intrinsic timer mechanism, rather than some extrinsic factor, determines the temporal sequence of the developmental events in the retina. Furthermore, this indicates that the intrinsic timer

mechanism may regulate progenitor cell maturation, a rather earlier developmental event in retina compared to cell fate determination. Consistent with this, ganglion cell development was initiated and propagated normally when small pieces of the peripheral region of chick retina were cultured without preexisting ganglion cells (McCabe et al., 1999). We have not pursued study of the intrinsic timer mechanism in the work described here. Although we can not eliminate this mechanism, the mechanisms for which we do have evidence do not require an intrinsic timer mechanism.

I-D.iv. Hedgehog (Hh) signaling

Introduction to Hh Signaling

The Hedgehog (Hh) signaling pathway regulates a wide range of developmental processes including proliferation, cell survival, patterning, and cell fate specification in many regions of developing embryos of invertebrate and vertebrate species. Hh is a secreted signaling molecule (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993), which was first identified by a large-scale screen for mutations that impair Drosophila development (Nusslein-Volhard and Wieschaus, 1980). Vertebrate species have three orthologs of Hh genes, Desert Hedgehog (Dhh), Indian Hedgehog (Ihh), and Sonic Hedgehog (Shh) (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994; Marigo et al., 1995) except zebrafish, which have three additional Hh orthologs, tiggywinkle hedgehog (TwHh) (Ekker et al., 1995), echidna hedgehog (Ehh) (Currie and Ingham, 1996), and qiqihar hedgehog (Qhh) (Ingham and McMahon, 2001) due to a whole-genome duplication occurred in the teleost fish lineage (Jaillon et al., 2004). Other components of the Hh signaling pathway include Patched (Ptc), the transmembrane receptor of the pathway, Smoothened (Smo),

another transmembrane protein important in the transduction of the Hh signaling, and a zinc-finger transcription factor (Ci in Drosophila, GLI1-3 in vertebrates) (Lee et al., 1992; Alcedo et al., 1996; Alexandre et al., 1996; Marigo et al., 1996; van den Heuvel and Ingham, 1996).

Generation and release of the active ligand involves multiple post-translational processing steps of the Hh protein (Burke et al., 1999; Chamoun et al., 2001; Ingham and McMahon, 2001; Caspary et al., 2002; Dai et al., 2002; Ma et al., 2002). Once the signal sequence is removed, its own C-terminal domain cleaves the Hh molecule between conserved glycine and cysteine residues, generating two molecules, a C-terminal domain with no known signaling activity and a cholesterol-modified N-terminal Hh signaling molecule (HhN) with size of approximately 19 kDa (Lee et al., 1994; Porter et al., 1996). Subsequently, a palmitic acid moiety (Pepinsky et al., 1998) is added to the N-terminus by the acyltransferase Skinny hedgehog (Ski, HHAT in humans) (Chamoun et al., 2001; Lee and Treisman, 2001; Buglino and Resh, 2008). The cholesterol modification of HhN molecule leads to tight association with the plasma membrane in the synthesizing cells. Therefore, secretion of Hh-N and its transport for long-range action require the function of additional proteins such as Dispatched (Disp), a 12-span transmembrane transport protein (Burke et al., 1999; Ma et al., 2002), and Heparan sulfate proteoglycans such as Dally and Dally-like (Bellaiche et al., 1998; Lin et al., 2000; Bornemann et al., 2004; Han et al., 2004; Koziel et al., 2004). Dally and Dally-like also affects Hh signaling by facilitating binding of Hh to cell surface (Nakato et al., 1995; Lum et al., 2003; Han et al., 2004).

Activation of the Hh signaling pathway involves interaction between Hh and Patched (Ptc), the transmembrane receptor of Hh. This interaction eventually leads to the activation of a zinc-finger transcription factor, Ci in Drosophila or GLI1-3 in the

vertebrate (Chen et al., 1999a, 1999b). The interaction between Hh and Ptc is facilitated by several accessory receptor such as Dally and Dally-like (Nakato et al., 1995; Lum et al., 2003; Han et al., 2004), which also function in Hh transport, and Cdo and Boc (iHog and boi in Drosophila) (Tenzen et al., 2006; Yao et al., 2006). In the absence of Hh, Ptc inhibits the function of Smo (Taipale et al., 2002). In this condition, the transcription factor Ci/GLI is cleaved by the ubiquitin E3 ligase slimb (beta-TrCP in vertebrates) generating the truncated transcription repressor form of Ci/GLI (Jiang and Struhl, 1998; Price and Kalder, 1999; Wang et al., 1999; Price and Kalderon, 2002; Jia et al., 2005), and the nuclear translocation of Ci/GLI is prevented (Chen et al., 1999a; Wang et al., 2000). Among GLI1-3, the vertebrate orthologs of Ci, GLI1 and GLI2 are responsible for activator functions (Bai and Joyner, 2001) whereas GLI3 functions as transcription repressor (Wang et al., 2000; Litingtung et al., 2002). GLI1 expression is induced by active Hh signaling, primarily to provide positive feedback and to prolong the cellular responses to Hh. The mechanism by which Ci/GLI activity is regulated in the absence or the presence of Hh appears to be highly divergent between the Drosophila and the vertebrate species (Huangfu and Anderson, 2006; Varjosalo and Taipale, 2007). The differences in the signal transduction downstream of Smo will not be discussed in detail here. One of the major differences lies in the finding that the primary cilium acts as a ‘signaling center’ (Simpson et al., 2009).

Function of Hh Signaling in Drosophila Eye Development

Despite the structural divergence, many aspects of invertebrate eye development are conserved in vertebrates. Thus, to understand the mechanism of propagation of the onset of neurogenesis in the vertebrate retina, the factor that drives the neuronal differentiation in Drosophila eye imaginal disc needs to be considered. In Drosophila eye

disc, Hh induces the initiation and the propagation of retinal differentiation. The differentiation of individual ommatidia in the Drosophila compound eye is initiated in the posterior margin of the eye imaginal disc and propagates in a wave progressing anteriorly (Tomlinson and Ready, 1987; Treisman and Heberlein, 1998). The front of differentiation, known as the morphogenetic furrow (MF), can be marked both by its morphology and by the changes in gene expression (Tomlinson and Ready, 1987; Wolff and Ready, 1991). The differentiation process is characterized by progressive changes in the expression pattern of atonal (ato), a Drosophila proneural gene. Initially, ato is expressed by ectodermal cells in a dorsoventral stripe across the disc in and just anterior to the MF. Subsequently, Ato expression becomes restricted first to clusters of a small number of cells and later to regularly spaced individual cells (Jarman et al., 1994, 1995; Dokucu et al., 1996). Individual ato expressing cells differentiate into R8 photoreceptors (Jarman et al., 1994) and orchestrate ormmatidial formation by inducing neighboring uncommitted cells to take the fate of other photoreceptor cell types (Freeman, 1994; Tio et al., 1994; Tio and Moses, 1997). Hh plays in pivotal role in the initiation and progression of ommaitidial differentiation by up-regulating ato expression (Heberlein et al., 1993; Ma et al., 1993; Heberlein and Moses, 1995; Borod and Heberlein, 1998; Dominguez, 1999; Greenwood and Struhl, 1999).

Drosophila compound eye and the vertebrate retina share similarities in key events of development. Analogous to the posterior-to-anterior propagation of ommatidial differentiation of Drosophila eye, neuronal differentiation is initiated in the center and then the area with differentiating cells expands progressively towards peripheral margin in the developing vertebrate retina (Dutting et al., 1983; Prada et al., 1991; Reese and Colello, 1992; McCabe et al., 1999). In addition, several key molecules or signaling pathways are also conserved among invertebrate and vertebrate species. For example,

the vertebrate orthologues of ato, required for R8 photoreceptor differentiation, are also implicated in neuronal differentiation in the vertebrate retina. Furthermore, beyond the front of neurogenesis in the vertebrate retina, Notch signaling inhibits neuronal differentiation in the cells neighboring the newly differentiating cells (Dorsky et al., 1995; Dorsky et al., 1997; Henrique et al., 1997; Scheer et al., 2001; Jadhav et al., 2006; Nelson et al., 2007), similar to the Notch-mediated lateral inhibition in the Drosophila eye disc. These similarities raise the possibility that Hh also plays a key role in driving the propagation of retinal differentiation in the vertebrate species. Several studies investigated the function of Sonic hedgehog (Shh), one of the vertebrate homologues of Hh, in the developing vertebrate retina. The following section will examine those studies in more detail.

Sonic Hedgehog (Shh) in the Developing Vertebrate Retina

Studies in the zebrafish retina first demonstrated that sonic hedgehog (Shh) is also required for normal progress of differentiation across the retina much as its Drosophila counterpart promotes the wave of differentiation in the Drosophila eye disc (Neumann and Nuesslein-Volhard, 2000; Stenkamp et al., 2000; Stenkamp and Frey, 2003; Masai et al., 2005). Shh is expressed by a subset of newly differentiating neurons in the zebrafish retina, and Shh expression itself propagates towards the periphery following the wave of ganglion cell differentiation (Neumann and Nuesslein-Volhard, 2000). Mutation in the Shh gene resulted in a delay in ganglion cell differentiation. Shh is also expressed by a subset of ganglion cells in other species including chick and mouse retina (Jensen and Wallace, 1997; Neumann and Nuesslein-Volhard, 2000; Zhang and Yang, 2001). Thus, it is likely that the function of Shh is common in other vertebrate species.

The mechanism by which Shh drives the front of ganglion cell development is yet to be understood. Ganglion cells are the first neuronal cell type generated in the retina (Rubinson and Cain, 1989; Snow and Robson, 1994; Belecky-Adams et al., 1996). Ganglion cells have been considered as a 'default' fate, and the presence of ganglion cells influences the environment so that the next cells to differentiate take a non-ganglion cell fate (Reh and Tully, 1986; Belliveau and Cepko, 1999; Waid and McLoon, 1998). Thus, the following model is possible. Shh, expressed by newly differentiating ganglion cells influence neighboring preneurogenic progenitor cells to switch their mode of division to neurogenic. Subsequently, progenitor cells begin to generate postmitotic cells, which differentiate as ganglion cells, the first cell type to develop in a region of retina. This possibility was tested in this thesis, and the results are presented in chapter II.

I.D.v. Fibroblast Growth Factor (FGF) Signaling

Introduction to FGF Signaling

Fibroblast growth factor (FGF) was initially identified as a substance that stimulates fibroblast to proliferate (Gospodarowicz, 1974). Later, FGFs were identified as a family of growth factors. In mouse, there are a total of 22 FGFs that can be classified into seven subfamily (Ornitz and Itoh, 2001; Itoh and Ornitz, 2004; Popovici et al., 2005). FGFs regulate multiple biological activities including many fundamental developmental events. In the vertebrate nervous system, FGFs participate in diverse cellular processes during development including neural induction, specification of regional identity, cell proliferation and differentiation (reviewed in Mason, 2007).

FGF signaling is activated by specific ligand-receptor binding. Four genes (FGFR1-4) encode receptor proteins for FGFs, the transmembrane receptor tyrosine kinases (RTKs) (Itoh and Ornitz, 2004), and they can be further diversified through alternative splicing. The interaction between FGFs and their specific receptors requires the presence of Heparan sulphate proteoglycans (HSPGs) as a co-factor (Ornitz and Itoh, 2001). Much as signaling pathways induced by other RTKs, FGFRs, upon activation, trigger the sequential activation of the multiple kinases including Raf, MEK (MAPK/ERK kinase), and mitogen-activated protein kinase [MAPK, or extracellular regulated kinase (ERK)] (reviewed in Powers et al., 2000). This pathway mediates majority of the known functions of FGFs during development. In addition, two other major branches of FGF signaling transduction exist (reviewed in Mason, 2007). FGF stimulation also activates phosphatidylinositol 3 (PI3) kinase, which in turn activates the Akt pathway. This mode of FGF signaling appears to mediate the anti-apoptotic effects of FGFs in the developing nervous system. FGFs can also mediate yet another signaling pathway involving phospholipase Cγ (PLCγ) and subsequent calcium release.

FGF Signaling in the Vertebrate Retina

FGF signaling also takes place in the developing retina. Studies have implicated FGF signaling in multiple events during the vertebrate retinal development. They include the specification of neural retinal fate, induction of neuronal differentiation, and cell fate specification (reviewed in Yang, 2004). One of the earliest functions of FGF signaling in retinal development is the patterning of the early optic vesicle. Active FGF signaling specifies neural retina and represses retinal pigmented epithelium (RPE) fate. FGF1 (previously known as acidic FGF or aFGF) and FGF2 (previously known as basic FGF or bFGF) are expressed in the surface ectoderm overlying the optic vesicle (de Iongh and

McAvoy, 1993; Pittack et al., 1997; Desire et al., 1998; Nguyen and Arnheiter, 2000), at a close proximity to the distal optic vesicle. The receptors, FGFR1 and FGFR2, are expressed in the developing optic vesicle (Wanaka et al., 1991; Tcheng et al., 1994). Gain- or loss of function studies showed that active FGF signaling in the distal optic vesicle induced specification of neural retina (Park and Hollenberg, 1989; Pittack et al., 1991; Guillemot and Cepko, 1992; Zhao et al., 1995; Pittack et al., 1997; Hyer et al., 1998; Nguyen and Arnheiter, 2000) and repressed RPE fate. This role is mediated, at least in part, by negative regulation of Mitf expression (Mochii et al., 1998b; Nguyen and Arnheiter, 2000), whose expression is required for RPE differentiation (Mochii et al., 1998a, 1998b). This is mediated by RTK signaling (Galy et al., 2002). In addition to FGF1 and 2, another FGF, FGF9, is expressed in the distal optic vesicle and appears to demarcate the boundary between the retina and the RPE (Zhao and Overbeek, 1999; Zhao et al., 2001).

Data suggest an additional function of FGF signaling in promoting the wave of ganglion cell differentiation. FGFs have been previously shown to be both necessary (Desire et al., 1998) and sufficient (Pittack et al., 1991; Guillemot and Cepko, 1992; Zhao et al., 1995) for ganglion cell differentiation. Significantly, inhibition of FGF signaling by blocking FGFRs retarded the progression of the front of ganglion cell differentiation, whereas treatment with FGF1 resulted in the premature differentiation of ganglion cells in chick retinal explant culture (McCabe et al., 1999). Another independent study reported that FGF3 and FGF8 are expressed in the center of the retina prior to the first appearance of ganglion cells and are necessary for initiation of ganglion cell development both in chick and in zebrafish (Martinez-Morales et al., 2005). As ganglion cells are the first neuronal type generated in the retina, the data reported above raise the possibility that FGF signaling drives progression of neuronal differentiation. Therefore,

FGF signaling was considered as a candidate factor that induces progenitor cell maturation. However, previous studies do not agree on the specific members of FGF involved in initiation of ganglion cell genesis and on the sources of FGF signaling. More careful characterization of FGF signaling molecules and their receptor in the developing retina must precede testing the role of FGF signaling in the progenitor cell maturation. Thus, role of FGF signaling was not studied in this thesis.

FGF signaling also has been implicated in other aspects of retinal development such as cell fate specification (McFarlane et al., 1998; Patel and McFarlane, 2000; Zhang et al., 2003) and maintenance of retinal stem cells (Tropepe et al., 2000; Fischer et al, 2002a, 2002b). Although these functions of FGF signaling are significant, they will not be further discussed as they are less relevant to this thesis.

I.D.vi. Delta-Notch Signaling

As discussed above (I.C.iv), the uniform expression of components of the Notch signaling system may account for the molecular characteristics that distinguish preneurogenic progenitor cells from neurogenic progenitor cells. Observations that the caudal stem zone of the spinal cord, analogous to the preneurogenic progenitor cells of the retina, has uniform expression of Delta1 and Notch1 support this view (Henrique et al., 1995; Caprioli et al., 2002; Akai et al., 2005). Consistent with the expression pattern of these genes, Delta-Notch signaling appears to play a role in maintaining the proliferation of caudal stem zone cells. Blocking Notch signaling by introducing dominant negative Delta1 resulted in reduced proliferation (Akai et al., 2005). A similar mechanism may account for the maintenance of preneurogenic mode of division in the developing retina. Therefore, the following hypothesis can be proposed. It is possible that Notch

signaling in the peripheral retina negatively regulates progenitor cell maturation and maintains the preneurogenic mode of division.

I.D.vii. Wnt Signaling

Introduction to Wnt Signaling

Signaling mediated by Wnt family proteins participate in diverse cellular processes during development including early embryonic patterning, cell proliferation, and cell fate specification. The Wnt family consists of a number of secreted proteins that signal through the cell surface receptor, Frizzled (Fz). There are multiple Wnt proteins and Fz. For instance, mouse has 19 Wnt proteins and 9 Fz. In the signal-sending cells, Wnt molecules undergo palmitoylation at a conserved cystein (Willert et al., 2003). Palmitoylation is critical for activation of the signaling pathway, although the mechanism is not completely understood (Willert et al., 2003).

Studies have characterized three main branches of the Wnt signal transduction pathway. In the canonical pathway, a secreted Wnt protein interacts with a receptor complex present on the surface of the signal-receiving cells comprising Fz, a seven-pass transmembrane protein (Bhanot et al., 1996), and its co-receptors, low density lipoprotein (LDL) receptor related protein 5 (LRP5) and LRP6, a single-pass transmembrane protein (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). Fz signals through heterotrimeric G protein activation (Slusarski et al., 1997; Liu et al., 2001; Katanaev et al., 2005). The interaction between the ligand and the receptor complex results in activation of Dishevelled (DVL), a cytoplasmic scaffold protein (Wharton, 2003). Without Wnt stimulation, a complex consisting of Axin, adenomatosis polysis coli (APC), glycogen synthase kinase 3β (GSK3 β) induces phosphorylation and

degradation of β-catenin. Activated DVL, however, disassembles this complex, which results in an accumulation of β-catenin in the cytoplasm. β-catenin, then, is translocated into the nucleus, forms a complex with T-cell specific transcription factor (TCF), and activate the transcription of Wnt-responsive genes (reviewed in Logan and Nusse, 2004).

Wnt mediates other signal transduction pathways distinct from the canonical pathway (reviewed in Ciani and Salinas, 2005). As Wnt2b, a major Wnt molecule expressed in the developing retina, signals through the canonical pathway to exert its function, the branches of Wnt pathway other than the canonical pathway will be discussed only briefly here. In the planar cell polarity (PCP) pathway, activated DVL signals to Rho GTPases. This pathway is responsible mainly for controlling the polarity of cells and tissues. Wnt/Calcium pathway also involves activation of DVL but signals through the release of intracellular calcium and the subsequent activation of calcium/calmodulin dependent kinase II (CaMKII) and the activation of protein kinase C (PKC). Wnt/Calcium pathway is implicated in cell fate and cell movement.

Wnt Signaling in the Vertebrate Retina

Signaling mediated by Wnt2b may negatively regulate progenitor cell maturation by maintaining the preneurogenic mode of division in the peripheral retina. Previous characterization of expression and function of Wnt2b supports this idea. Wnt2b is expressed in a small region at the peripheral margin of the optic cup (Jasoni et al., 1999; Kubo et al., 2003; Liu et al., 2003). Active canonical Wnt signaling was monitored by the activation of a reporter gene under the control of Wnt responsive elements or by the expression of Lef1, one of the target genes of canonical Wnt signaling. Canonical Wnt signaling was active in the periphery of the early embryonic retina, located next to the source of Wnt2b, but it was inactive in the center of the retina where cells were

differentiating (Kubo et al., 2003; Liu et al., 2003). Other Wnt molecules are also expressed in the embryonic vertebrate retina (Liu et al., 2003). However, Wnt2b appears to be the only Wnt that mediates activation of the canonical pathway in the peripheral region of the developing retina as the other Wnt molecules are expressed in the center of the retina (Liu et al., 2003). Thus, the region of the active canonical Wnt signaling is consistent with the possibility that signaling mediated by Wnt2b maintains the preneurogenic progenitor cells.

Studies have reported the mitogenic effect of some Wnt molecules in other regions of the central nervous system including spinal cord (Megason and McMahon, 2002) and cerebral cortex (Chenn and Walsh, 2002). Wnt2b signaling also appears to have a similar mitogenic function in the developing retina (Kubo et al., 2003, 2005; Kubo and Nakagawa, 2009). Prolonged expression of Wnt2b in the embryonic chick retina inhibited neuronal differentiation whereas blocking canonical Wnt signaling by expressing a dominant negative form of Lef1 resulted in premature differentiation (Kubo et al., 2003). Furthermore, cell proliferation was facilitated when cells dissociated from peripheral region of the early embryonic chick retina were cultured in Wnt2b conditioned medium (Kubo et al., 2003). Collectively, these findings suggest that Wnt2b signaling promotes cell proliferation in the peripheral retina. They also raise the possibility that Wnt2b signaling maintains the preneurogenic mode of division.

Another study, however, suggested a different function of Wnt2b signaling. Manipulating Wn2b signaling using a retroviral vector in chick retina showed that canonical Wnt signaling mediated by Wnt2b and β-catenin specifies the peripheral structures of the eye including iris and ciliary body (Cho and Cepko, 2006). The premature neuronal differentiation induced by expression of dominant negative form of Lef1 (Kubo et al., 2003) was previously interpreted that canonical Wnt signaling normally

maintains cell proliferation and inhibits neuronal differentiation. However, another interpretation is also possible. The region, which normally would be specified as iris and ciliary epithelium, may have been reprogrammed towards the neural retina as a result of inhibition of canonical Wnt signaling.

To resolve the inconsistency among the suggested roles of Wnt2b, preliminary studies were done. The results were most consistent with Wnt2b specifying peripheral eye structures, which are not presented here. It has been suggested that the fate of the peripheral structures of the eye is specified early during the development (Dhawan and Beebe, 1994; Kubota et al., 2004). However, the boundary between presumptive ciliary epithelium and neural retina may not be fixed until later stages. Several genes such as collagen IX are expressed both in the ciliary epithelium and in the peripheral region of the early developing neural retina (Kubo et al., 2003). Collagen IX expression was used to identify retinal progenitor cells or to identify the presumptive ciliary body by different groups, which may have resulted in inconsistency in the interpretation of the data. However, Wnt2b function in maintaining the preneurogenic progenitor cells in the peripheral retina is still an open possibility. A more recent study suggested that Hes1 mediates the role of Wnt2b in maintaining cell proliferation in the peripheral region of the developing retina, independent of Notch signaling (Kubo and Nakagawa, 2009). This finding suggests the possibility that Wnt2b has a dual function, specification of iris and ciliary body and maintaining cell proliferation, and that the two functions are mediated by different downstream effectors. Consistent with this, the region of active Wnt2b signaling, as indicated by Lef1 expression, encompasses broad regions including both the presumptive ciliary epithelium and the peripheral region of the developing neural retina (Kubo et al., 2003). Further investigation is needed to resolve the role of Wnt2b in the developing retina.

I.D.viii. Hypothesis

The second aim of this thesis is to understand the mechanisms underlying progenitor cell maturation from preneurogenic to neurogenic. Although initial maintenance of preneurogenic progenitor cells and later maturation of progenitor cells from preneurogenic to neurogenic are a well documented phenomenon, little is known about the underlying mechanism. The function of multiple factors, including a cell intrinsic timer mechanism and signaling pathways mediated by Hh, FGF, Notch, and Wnt, were evaluated.

Based on the data available from previous work on the candidate factors, the following hypothesis is proposed. Notch signaling in the peripheral region of the retina maintains the preneurogenic mode of progenitor cell division and thereby negatively regulates progenitor cell maturation. In addition, Shh is involved in the positive regulation of progenitor cell maturation and thus promote the propagation of neurogenesis. This hypothesis was tested in this thesis, which is presented in chapter II.

I.E. Initiation of Neurogenesis

I.E.i. Issue

Following the switch of the mode of division from preneurogenic to neurogenic, retinal progenitor cells initiate a neurogenic program and begin to generate neuronal and glial cell types. The factors involved in this process remain to be elucidated. Proneural genes, which encode class II HLH family transcription factors, have been implicated in the initiation of neuronal differentiation in many regions of the vertebrate central nervous system. However, their role in initiation of neurogenesis in the vertebrate retina is incompletely understood.

I.E.ii. Initiation of Neurogenesis in Drosophila Eye Imaginal Disc

Despite the apparent difference in structure, many aspects of Drosophila eye development are conserved in the vertebrate retina. Therefore, in order to address the question of what initiates neurogenesis in the vertebrate neural retina, the mechanism of initiation of neurogenesis in the Drosophila eye was examined.

Atonal, one of the proneural genes in Drosophila (Jarman et al., 1993, 1994; Goulding et al., 2000), plays a pivotal role in initiation of neurogenesis in Drosophila eye imaginal disc (Jarman et al., 1994). Ato functions in promoting neuronal differentiation in two consecutive steps. Ato is initially expressed uniformly by ectodermal cells in a dorsoventral stripe across the eye disc just ahead of the morphogenetic furrow (i.e. the front of differentiation in the Drosophila eye imaginal disc). This initial broad expression of ato specifies the ectodermal cells as neuronal progenitor cells (Jarman et al., 1994). Subsequently, ato expression becomes refined into evenly spaced isolated cells by lateral inhibition mediated by Notch signaling (Jarman et al., 1994). The ato positive,

isolated cells develop as R8 photoreceptor cells (Jarman et al., 1994), which induce generation of other photoreceptor cell types from neighboring uncommitted neural progenitor cells (Tomlinson and Ready, 1987; Ready, 1989; Banerjee and Zipursky, 1990). A null mutation in the ato gene resulted in complete loss of photoreceptors, and photoreceptor cell development was partially rescued by reintroducing ato expression induced by heat shock (Jarman et al., 1994). Generation of other photoreceptor cell types did not directly require autonomous ato expression, but was dependent on the normally developed R8 photoreceptor cells.

I.E.iii. Initiation of Neurogenesis in the Vertebrate Retina

Homologues of Drosophila ato are expressed in the vertebrate neural retina, raising the possibility that one or more of those genes function in initiation of neurogenesis. Consistent with this, Atoh7, a proneural gene expressed in the retina that is most closely related to ato, is a key factor for development of retinal ganglion cell. Genetic deletion of Atoh7 resulted in severe loss of ganglion cells (Brown et al., 2001; Liu et al., 2001). In addition, Atoh7 promotes expression of other genes that are involved in ganglion cell differentiation (Hutcheson and Vetter, 2001; Liu et al., 2001). Ganglion cells are the first neuronal type generated in the retina. It is possible that Atoh7 initiates the neurogenic program, much as ato functions in Drosophila eye disc, and that the resulting postmitotic neurons differentiate as ganglion cells. However, several lines of evidence suggest otherwise. Misexpression of Atoh7 in the developing retina failed to initiate neurogenesis efficiently (Liu et al., 2001). Also, Atoh7 was found to be expressed after a cell withdraws from the mitotic cycle and begins to differentiate (Yang et al., 2003). These findings suggest that an additional factor acts independently or upstream of Atoh7 to initiate neurogenesis in the vertebrate retina.

Other proneural genes are also expressed in the developing vertebrate retina. These include Ascl1 (Guillemot and Joyner, 1993; Jasoni and Reh, 1996), Neurod1 (Sommer et al., 1996; Acharya et al., 1997; Kanekar et al., 1997; Roztocil et al., 1997; Korzh et al., 1998), Neurod4 (Roztocil et al., 1997; Takebayashi et al., 1997), Neurog2 (Gradwohl et al., 1996; Sommer et al., 1996; Brown et al., 1998). Although much effort has been made towards understanding the role of these genes in cell fate specification (reviewed in Hatakeyama and Kageyama, 2004; Ohsawa and Kageyama, 2008), their role in initiation of neurogenesis has not been fully tested. Nevertheless, studies in other tissues of the vertebrate central nervous system make it likely that proneural genes also play a role in initiation of neurogenesis in the vertebrate retina. Our current understanding of the role of proneural genes in the developing nervous system is examined further in the following sections.

I.E.iv. Proneural Genes, Class II HLH Family Transcription Factors

Introduction to Proneural bHLH Transcription Factors

The vertebrate proneural genes were first identified as homologues of atonal and achaete-scute complex genes, the Drosophila proneural genes that promote neurogenesis (Lee, 1997; Guillemot, 1999). They are classified as class II HLH family transcription factors, one of the subclasses of the HLH family (reviewed in Murre et al., 1994). Similar to class I HLH family members (I.C.iii), class II HLH proteins share a sequence homology in the basic domain and the helix-loop-helix domain (reviewed in Bertrand et al., 2002). These domains are responsible for sequence specific DNA-binding and dimerization with another HLH family protein, respectively. Proneural genes can be classified further into several subgroups including ato family, Neurod family,

Neurogenin family, Achaete-scute family, and Olig2 family based on the sequence similarities within the bHLH domain. Most of these factors are transcription activators with exception of Olig2, which has transcription repressor activity (Mizuguchi et al., 2001; Novitch et al., 2001).

Biochemical Properties of Proneural bHLH Transcription Factors

Proneural bHLH transcription factors exert an effect on transcription. Through the basic domain, proneural factors bind to the hexanucleotide motif CANNTG, known as an E-box. Binding to DNA involves the direct interaction between basic domain and major groove of DNA and is required for the transcriptional regulation. Although these factors recognize four conserved bases of the E box, they must recognize additional flanking bases to confer the DNA-binding specificities and functional diversity. The additional interaction sites have not been fully characterized. bHLH transcription factors interact with each other via helix-loop-helix (HLH) domain. Formation of homo- or heterodimers between class I and class II factors is necessary for their activity as a transcription regulator (Johnson et al., 1992; Massari and Murre, 2000).

Function of Proneural bHLH Transcription Factors

Genetic studies showed that the vertebrate proneural bHLH transcription factors have similar proneural activity as their Drosophila counterparts. Null mutation of Ascl1 or Neurog2 resulted in severe defects in neurogenesis in many regions of the developing nervous system (Guillemot and Joyner, 1993; Fode et al., 1998; Casarosa et al., 1999; Horton et al., 1999; Ma et al., 1999), suggesting that their normal function may be to initiate neurogenesis. Proneural bHLH factors can induce cell cycle withdrawal and promote expression of genes required for differentiation. Transient expression of proneural bHLH factors in P19 cells resulted in neurogenesis, and the neuronal

differentiation was always preceded by up-regulation of expression of the cyclin-dependent kinase inhibitor, $p27^{Kip1}$ (Farah et al., 2000). Similarly, forced expression of Neurog2 in the developing spinal cord resulted in both premature cell cycle withdrawal and neuronal differentiation (Novitch et al., 2001). Expression of proneural bHLH factors is transient (Ben-Arie et al., 1996; Gradwohl et al., 1996; Ma et al., 1996), and the proneural activity usually involves successive up-regulation of the bHLH factors (Ma et al., 1996, 1998; Fode et al., 1998; Perron et al., 1998; Cau et al., 2002). Some bHLH factors can directly activate expression of genes encoding a functional protein for particular neurons. For instance, Atoh7 directly induces expression of the β3-subunit of the neuronal acetylcholine receptor (Matter-Sadzinski et al., 2001).

Another essential role of proneural bHLH factors is to restrict proneural activity to isolated progenitor cells. This process, known as 'lateral inhibition' is achieved by activation of Notch signaling. Proneural bHLH genes induce expression of Delta, the Notch ligand (Chitnis and Kintner, 1996; Fode et al., 1998; Casarosa et al., 1999; Ma et al., 1999; Cau et al., 2002), and therefore activate Notch signaling in the adjacent cells. Active Notch signaling results in expression of Hes genes such as Hes1 and Hes5 (Nishimura et al., 1998), which in turn repress proneural gene expression (reviewed in Kageyama et al., 2007). Through this mechanism, a small number of single cells are selected to undergo neuronal differentiation from the otherwise equivalent progenitor cells. This is particularly important to maintain progenitor cells for a period of development of the nervous system.

Proneural bHLH transcription factors also play a role in cell fate specification. Despite their common proneural activity, proneural bHLH factors are often expressed in distinct progenitor domains that are correlated with particular neuronal lineages. Furthermore, genetic deletion of each proneural gene resulted in loss of discrete types of

neurons. For instance, Math1, Neurog2, and Ascl1 direct specification of distinct interneurons in the spinal cord as indicated by their expression pattern and mutational analyses in mouse (Bermingham et al., 2001; Gowan et al., 2001). In the retina, individual or combinations of proneural bHLH factors are also linked to the specification of retinal cell types (Hatakeyama and Kageyama, 2004; Ohsawa and Kageyama, 2008). Furthermore, proneural genes regulate the transition from neurogenesis to gliogenesis in many regions of central nervous system by promoting neuronal fate and inhibiting glial fate (Cai et al., 2000; Tomita et al., 2000; Nieto et al., 2001; Vetter, 2001; Zhou et al., 2001; Inoue et al., 2002).

Regulation of Activity of Proneural bHLH Transcription Factors

The activity of proneural bHLH transcription factors is subject to regulation by other members of HLH transcription factor family. Positive or negative regulation of proneural bHLH transcription factors is essential for control of the normal period of neurogenesis, maintenance of neural progenitor cells, and promotion of gliogenesis. As discussed above, dimerization with class I HLH proteins is important for efficient transcriptional activity of the proneural proteins. In addition, function of proneural factors can be regulated negatively by other members of HLH transcription factor family such as class V and class VI HLH factors.

Members of class V HLH proteins, also known as Id (Inhibitor of differentiation), inhibit the function of proneural bHLH transcription factors and thereby regulate neurogenesis negatively. This class of HLH proteins includes Drosophila emc (extra macrochaetae) and four vertebrate homologues, Id1 to Id4 (Benezra et al., 1990; Ellis et al., 1990; Garrell and Modolell, 1990). These proteins have a highly conserved HLH domain but lack an adjacent basic domain that is required for DNA binding (Benezra et

al., 1990). They bind to E proteins (class I HLH proteins) with high affinity via the HLH domain. Thus these class V proteins can compete with proneural bHLH factors by forming heterodimers that lack DNA binding ability (Massari and Murre, 2000; Campuzano, 2001; Yokota et al., 2001).

Class VI HLH proteins also negatively regulate the function of proneural bHLH transcription factors. Drosophila Hairy and Enhancer of split [E(spl)] genes and their vertebrate homologues, Hes genes, constitute this class of HLH factors (Akazawa et al., 1992; Sasai et al., 1992; Feder et al., 1993). Among the seven members of the Hes family, Hes1 to Hes7, Hes1, Hes3, and Hes5 are expressed by progenitor cells in the developing vertebrate nervous system (reviewed in Kageyama et al., 2007). The inhibitory function of Notch signaling in neuronal differentiation is known to be mediated by the function of Hes1 and Hes5 (Nishimura et al., 1998). There are also bHLH genes that are related to Hes such as Hesr (or also called as Hey) (Iso et al., 2001) and Heslike (Miyoshi et al., 2004).

Members of Hes family HLH proteins share three conserved domains. First, they have a conserved bHLH domain. This is similar to that found in class I and class II HLH proteins. Unlike most other bHLH factors, however, Hes genes have the conserved proline residue in the middle of their basic region, which is suggested to be important for DNA binding specificity. Hes proteins bind to the CACNAG and the CACGCG sequence, which are called the N box and class C site, respectively. They bind these sites with a higher affinity than to E box (Sasai et al., 1992; Chen et al., 1997). Hes proteins also form a homodimer or a heterodimer with other Hes-related proteins through HLH domain (Iso et al., 2001). Second, the Orange domain, located just downstream of the bHLH domain, is also conserved among Hes genes. This domain consists of two amphipathic helices, and confers specificity for bHLH factor interactions (Dawson et al., 1995;

Taelman et al., 2004). It was also shown to mediate transcriptional repression (Castella et al., 2000) probably through interaction with a corepressor. Third, the WRPW domain is located at the carboxyl terminus and functions as a repressor domain. The repressor activity comes from the interaction with the corepressor TLE/Grg (Groucho related gene), a homologue of the Drosophila protein Groucho (Paroush et al., 1994; Fisher et al., 1996; Grbavec and Stifani, 1996).

Hes proteins inhibit proneural bHLH factors by two distinct mechanisms. In active repression, a homo- or heterodimer of Hes proteins binds to the N box or the class C site. Subsequently, the corepressor Grouch/TLE/Grg interacts with the WRPW domain and recruits the histone deacetylase, Rpd3. The resulting chromatin modification represses the transcription of the target gene. In addition, Hes proteins can inhibit the function of the proneural bHLH factors through passive repression. Hes proteins can form a heterodimer with other bHLH factors such as Ascl1 and E47 that normally bind to the E box. These heterodimers, however, can not bind to DNA and thus act as dominant-negative for the proneural activity (Kageyama et al., 2007). Consistent with their function in inhibition of proneural genes, Hes proteins are important for correct control of the initiation of neurogenesis, maintenance of neural progenitor cells, and promotion of gliogenesis.

Proneural bHLH Transcription Factors in the Developing Vertebrate Retina

The expression and function of proneural bHLH transcription factors have been characterized in the developing vertebrate retina. As introduced above, multiple proneural genes are expressed by progenitor cells and newly formed postmitotic cells in the retina during development. However, most studies have focused on understanding the function of proneural genes in cell fate specification (reviewed in Hatakeyama and

Kageyama, 2004; Ohsawa and Kageyama, 2008). Their role in initiation of neurogenesis has not been studied. Nevertheless, several lines of evidence suggest a potential role of proneural genes in neuronal differentiation in the retina. First, the onset of expression of proneural genes appears to precede the first appearance of postmitotic cells (Brown et al., 1998; Matter-Sadzinski et al., 2001). Second, a lineage tracing study revealed that Neurog2 expressing cells differentiate to generate all major retinal cell types (Ma and Wang, 2006). Third, when ectopically expressed in the retinal pigmented epithelial cells cultured in vitro, Neurog2 (Yan et al., 2001) or Neurod1 (Yan and Wang, 2000a, 2000b), was sufficient to generate multiple retinal cell types. Together, these studies suggest the possibility that one or more of the proneural genes expressed in the embryonic retina may play an important role in initiating neurogenesis.

I.D.viii. Hypothesis

The third aim of this thesis is to understand the mechanisms by which neurogenesis is initiated. As discussed above, proneural genes, members of class II bHLH transcription factor family, have been implicated in initiating neurogenesis in Drosophila eye disc. Given much similarity in the key mechanisms in development of Drosophila and the vertebrate retina, it is hypothesized that the vertebrate proneural genes also play a role in initiation of neurogenesis in the retina. This hypothesis was tested in this thesis, and the results are presented in chapter III.

CHAPTER II.

PROGENITOR CELL MATURATION IN THE VERTEBRATE RETINA

II.A. Introduction

Progenitor cells in the early developing vertebrate nervous system divide so that each division produces two cells that divide again. This early, preneurogenic mode of cell division results in an exponential increase in the number of progenitor cells. The entire nervous system is derived from a relatively small number of progenitor cells. Thus, preneurogenic divisions are essential for growth of the nervous system within the finite period of normal development. Later in development, progenitor cells change their mode of division so that one or both cells produced by a division can withdraw from the mitotic cycle and differentiate. This later, neurogenic mode of cell division is essential for generating a functional nervous system. The fundamental molecular differences between preneurogenic and neurogenic progenitor cells and the mechanisms that control the conversion of progenitor cells from preneurogenic to neurogenic are incompletely understood.

The vertebrate retina, part of the central nervous system, is a useful model for studying the difference between preneurogenic and neurogenic progenitor cells. All cells of the optic vesicle and early optic cup are preneurogenic progenitors, and no differentiation occurs. The first cells to differentiate are in the center of the neural retina (Prada et al., 1991; Reese and Colello, 1992; McCabe et al., 1999). For a period of development, cell division gives rise to cells that differentiate in central retina, and at the same time, the progenitor pool continues to expand without differentiation in the periphery of the retina (Dutting et al., 1983). The border between neurogenic and preneurogenic progenitor cells, the neurogenic front, progresses more peripherally in the retina until neurogenesis is taking place across the entire neural retina. In developing rodents, the period between the onset of neurogenesis and the time at which

neurogenesis is taking place across the entire retina is approximately a half day (Reese and Colello, 1992). In chick embryos, however, progenitor cells in the periphery of the retina are preneurogenic while those more central are neurogenic for a period of several days (Prada et al., 1991; McCabe et al., 1999). Based on their different positions, preneurogenic and neurogenic progenitor cells can be easily studied in the same retina during this period of chick development.

In the present study, we used developing chick retina to address two questions. First, what are the fundamental molecular differences between preneurogenic and neurogenic progenitor cells, and second, what controls the transition from preneurogenic to neurogenic states? We show that preneurogenic progenitor cells in the retina, unlike neurogenic progenitor cells, express the Notch ligand, Delta1. E2A, a binding partner for proneural bHLH transcription factors required for neuronal differentiation (Johnson et al., 1992; Roberts et al., 1993; Shimizu et al., 1995; Fode et al., 2000), is expressed exclusively by neurogenic progenitor cells. During the preneurogenic-to-neurogenic transition, progenitor cells down-regulate expression of Delta1 and up-regulate expression of E2A. Interrupting Notch signaling experimentally in the preneurogenic population resulted in loss of Delta1 expression, up-regulation of E2A expression and the premature onset of neurogenesis. Sonic hedgehog (Shh) is expressed normally by a subset of newly differentiating ganglion cells. Misexpression of Shh in the preneurogenic population locally initiated the preneurogenic to neurogenic transition including the down-regulation of Delta1 expression.

II.B. Experimental Procedures

Animals

Pathogen-free, fertilized White Leghorn chicken eggs were obtained from Hy-line (Spencer, Iowa). Eggs were incubated at 37°C and 98% relative humidity.

Dissociated retinal cell culture

Retinas from E3.5 embryos were removed from the eyes in CMF-HBSS. The central or peripheral third of each retina was dissected. The central or peripheral retinal fragments were incubated in trypsin/EDTA for 15 min at 37ºC. F12 medium containing 15% fetal bovine serum was added, and the cells were dissociated by gentle trituration. Cells were plated at low density on 25 µg/ml laminin-coated coverslips in defined F12 medium supplemented according to Bottenstein et al. (1980), and cultured at 37ºC in 5% CO2 for 24 hrs.

Immunocytochemistry

Embryonic chick retinas were processed for immunohistochemistry as described previously (Waid and McLoon, 1995). The primary antibodies used recognized the chick RA4 antigen (McLoon and Barnes, 1989), chick Delta1 (a gift from David Ish-Horowicz, University of Oxford; Henrique et al., 1997), chick Notch-1 (a gift from Yoshio Wakamatsu, Tohoku University), mouse Notch-1 (a gift from Urban Lendahl, Karolinska Institute; Wakamatsu et al., 1999), chick Shh (from the Developmental Studies Hybridoma Bank, University of Iowa), chick Islet1 (from the Developmental Studies Hybridoma Bank, University of Iowa), human Sox2 (from R&D Systems, Minneapolis), human neural protein HuC/D (from Molecular Probes, Eugene), proliferating cell nuclear antigen (PCNA; from Sigma Chemical Co., St. Louis), phospho-histone-H3 (from

Upstate Cell Signaling Solutions, Lake Placid) and human Ki67 antigen (from Dako, Denmark). Secondary antibodies used were affinity purified donkey anti-mouse IgG, donkey anti-rabbit IgG, and donkey anti-goat IgG, each conjugated to Cy2 or Cy3 (Jackson Immunoresearch Labs Inc., West Grove). Sections were counterstained with DAPI.

In Situ Hybridization

Eyes were fixed and prepared for in situ hybridization as for immunohistochemistry (Waid and McLoon, 1995). Chromogenic in situ hybridization was performed on frozen sections as described previously (Tuttle et al., 1999). For double fluorescence in situ hybridization combined with immunohistochemistry, frozen sections were simultaneously hybridized with digoxigenin- and fluorescein-labeled RNA probes, and each probe was detected sequentially according to Fior and Henrique, 2005. First, fluorescein-labeled RNA probes were detected with anti-fluorescein antibody conjugated with alkaline phosphatase (from Roche, Indianapolis) and visualized by an enzymatic reaction with Fast Red substrate (from Roche, Indianapolis). Subsequently, digoxigenin-labeled RNA probes were detected with anti-digoxigenin antibody conjugated with peroxidase (from Roche, Indianapolis) and developed using TSA^{TM} Plus Fluorescein System (from Perkin-Elmer, Waltham). Immunohistochemistry was performed after the in situ hybridization. cDNAs in plasmid vectors were used as templates to synthesize the RNA probes. The following cDNAs were used: Delta1 (a gift from David Ish-Horowicz, Oxford University), Sox2 (a gift from Paul Sharpe, King's College), Neurod1 (a gift from Elise Lamar, Salk Institute), Isl1 (a gift from Sam Pfaff, Salk Institute), E2A (a gift from Klemens Meyer, University of Cambridge), and WFDC1 (a gift from Constance Cepko, Harvard University).

Microscopy

Digital images of histological slides were captured from an epifluorescence microscope. Five to 10 images of each field were captured at regularly spaced planes of focus. Images for each wavelength were captured separately. Images were deconvolved using the Microtome (VayTek) within Image-Pro Plus image-processing program (Media Cybernetics). A stack of deconvolved images for each wavelength was combined into a single image, and the combined images for each wavelength were assigned to different colors in a single pseudo-colored image for each field of view. A minimum of 6 eyes were examined for each age and experimental condition. All numerical data are expressed as the average plus-or-minus the s.e.m. Statistical significance was evaluated using Student's T-test.

Manipulation of Notch or Shh Signaling

Antisense oligonucleotides were administered in vivo to knockdown expression of Notch or Delta1 in the developing retina using methods described previously (Austin et al., 1995; Ahmad et al., 1997). Missense oligonucleotides with conservative rearrangements of the antisense sequences were used as controls. All oligonucleotides were synthesized with phosphorothioate linkages between bases. The eyes of E3.5 or E4.5 embryos were injected with up to 1 µl of an oligonucleotide so that the final oligonucleotide concentration inside the eyes was approximately 25 µM. This concentration of oligonucleotide resulted in no detectable change in retinal cell death as determined by TUNEL assay. The effectiveness of the antisense oligonucleotide treatments for knockdown of protein expression was verified by western blot analysis (not shown). In other embryos, one eye was injected with a γ-secretase inhibitor, DAPT (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; from Sigma-

Aldrich), to disrupt Notch signaling as described previously (Geling et al., 2002; Nelson et al., 2006). One microliter of 1 μM DAPT in DMSO was injected. This dose of DAPT resulted in a small increase in retinal cell death, whereas higher concentrations of DAPT resulted in considerable cell death and unhealthy retinas.

DNA expression vectors were introduced into retinal progenitor cells in vivo by in ovo electroporation as described previously (Muramatsu et al., 1996; 1997). Briefly, a plasmid expression vector was injected into the vitreous of one eye at E3.5. The vectors used were pCX-IG-Shh for misexpression of Shh (Riddle et al., 1993) or pCS2-Su(H)DBM for expression of a dominant negative Su(H) to block Notch signaling (Wettstein et al., 1997). A reporter construct, pCAG-nls-EGFP, was co-injected in each case to label transfected cells or alone as a control. Approximately 300 ng of each vector mixed with Fast Green was injected into an eye. Electrodes were placed on both sides of injected eyes, and three 50 milliseconds electric pulses were applied at 25 V with 950 milliseconds interpulse intervals.

Treated embryos were incubated for an additional 24 hours, after which the retinas were analyzed by immunohistochemistry and in situ hybridization as described above.

II.C. Results

II.C.i. Differentiation Markers Distinguish Distinct Preneurogenic and Neurogenic Zones in the Developing Chick Retina

The spatiotemporal pattern of the onset of neurogenesis was mapped for the developing chick retina. Sections of retinas at different stages of development were processed for immunohistochemistry with an antibody to HuC/D, transcription factors expressed by some differentiating retinal cells (Ekstrom and Johansson, 2003), or with the RA4 antibody, which recognizes a microtubule-associated protein expressed by newly differentiating retinal ganglion cells (McLoon and Barnes, 1989; Waid and McLoon, 1995), the first cell type born in the retina. We used the presence of cells labeled for RA4 or HuC/D to define the neurogenic zone of the retina. At stage 14 (embryonic day 2 or E2), just after formation of the optic cup, no cells were differentiating (Fig. 1*A*). Differentiating cells were first detected near the center of the retina at stage 16 (E2.5; Fig. 1*B*). As development progressed, the region of the retina with differentiating cells, the neurogenic zone, expanded and continued to be surrounded peripherally by a region without differentiating cells, the preneurogenic zone (Fig. 1*B-E*, 2). By stage 27 (E5), the preneurogenic zone had split into two areas, a smaller area at the ventral-temporal margin and a larger area encompassing most of the nasal and dorsal periphery (Fig. 2). By stage 29 (E6), the preneurogenic zone was restricted to a small region on the nasal side of the retina. The nasal side is larger than the temporal side in the adult retina. This asymmetry was first apparent at stage 31 (E7). The prolonged presence of the preneurogenic zone on the nasal side where the progenitor cell population is increasing exponentially may be responsible for the differential growth of the two sides of the retina. By stage 34 (E8), a preneurogenic zone could no longer be identified, and the entire

neural retina contained differentiating cells. The ciliary zone, the most peripheral margin of the optic cup, was identified by expression of WFDC1 (Fig. 1*Eb*; Rowan et al., 2004), and it never exhibited neuronal differentiation. The ciliary zone contributes to formation of the ciliary body and iris. The spatiotemporal pattern of the onset of neurogenesis reported here is similar to that described previously for the chick retina (Dutting et al., 1983; Prada et al., 1991; McCabe et al., 1999).

It is important to recognize that at the developmental ages studied here, the majority of cells across the entire retina were dividing. Later in development, cell genesis will be complete in the central region of the retina, and the proliferative zone will be restricted to the peripheral margin of the retina. In many cold blooded vertebrates, the retina continues to grow at its peripheral margin while the rest of the retina is completely mature and functioning (Perron et al., 1998). This growth zone sits next to the ciliary body and has been named the ciliary marginal zone (CMZ). The CMZ includes a preneurogenic region where the progenitor pool is maintained, and a neurogenic region where differentiating cells are produced. We study developing chick retina before the ciliary body has formed, so that nothing we study is next to the ciliary body. The preneurogenic and neurogenic zones in chick retina together are functionally equivalent to the CMZ described for cold blooded vertebrates.

II.C.ii. Sox2 Is Expressed by Neural Retinal Progenitor Cells

We next began to look for differences between progenitor cells in the preneurogenic and neurogenic zones that could explain their different modes of division. It is possible that progenitor cells in the preneurogenic zone have not developed the competency to generate neurons. We tested the competence of progenitor cells in the preneurogenic zone to generate neurons using dissociated cell culture. Previous studies showed that

culturing dissociated embryonic retinal cells promoted differentiation (Reh and Kljavin, 1989; Guillemot and Cepko, 1992). These previous studies, however, used retinas that were mostly neurogenic, and it is possible that only these cells differentiated in culture. We dissociated cells isolated from the preneurogenic zone of E3.5 embryonic chick retina, taking care to exclude cells from the central retina where cells were already differentiating. The dissociated preneurogenic progenitor cells were cultured at low density for 24 hours and then processed for immunocytochemistry using the RA4 or HuC/D antibodies. In cultures with cells plated at low density so that few cells were in contact with one another, most cells expressed differentiation markers. Typically, isolated cells and cells in small groups were differentiating, while fewer cells in larger clumps expressed the differentiation markers (Fig. 3). This result shows that progenitor cells from the preneurogenic zone are capable of differentiation. It also suggests that cell-cell contact can prevent differentiation.

We also examined Sox2 expression in the preneurogenic and neurogenic progenitor cells. Sox2, an HMG-domain transcription factor, is expressed by neural progenitor cells in many regions of the developing central nervous system, and Sox2 expression has been linked to the competence of progenitor cells to generate neurons (Pevny and Placzek, 2005; Taranova et al., 2006). Sox2 expression in the early developing retina was examined by immunohistochemistry and in situ hybridization. The presence of RA4-positive differentiating cells was used to define the neurogenic zone of the retina. At stage 14 (E2), prior to the onset of differentiation, virtually all cells in the future neural retina expressed Sox2 (Fig. 1*A*). From stage 16 (E2.5) on, all retinal progenitor cells in both the preneurogenic and neurogenic zones expressed Sox2 (Fig. 1*B-D, Ec, Fc, Gc*). It also was noted that Sox2 is expressed by lens progenitor cells throughout development (Fig. 1). Sox2 was not expressed in the most peripheral margin, the ciliary

zone (Fig. 1*B-D, Ec*). Sox2 expression also was excluded from the layers of retina with accumulations of post-mitotic, differentiating cells. For example, at stage 30 (E6.5), Sox2 expression was largely absent from ganglion cell and photoreceptor cell layers as defined by expression of Islet1 and Neurod1, respectively (Fig. 4). This is consistent with previous studies showing that Sox2 is expressed only by proliferating cells and that it is down-regulated as cells begin to differentiate (Pevny and Placzek, 2005; Taranova et al., 2006). As with the dissociation experiment, expression of Sox2 by preneurogenic progenitor cells provides evidence that these cells are competent to generate neurons.

II.C.iii. Delta1 Is Expressed by Preneurogenic Progenitor Cells and Not by Neurogenic Progenitor Cells

The Notch signaling pathway has been linked to control of cell division and differentiation in the developing retina (Dorsky et al., 1995, 1997; Henrique et al., 1997; Scheer et al., 2001; Jadhav et al., 2005; Nelson et al., 2006). This evolutionarily conserved pathway includes Notch, a transmembrane receptor expressed on the cell surface, and several cell surface ligands for Notch, including Delta1 (also called Delta-like1 or Dll1; reviewed by Campos-Ortega, 1995; Artavanis-Tsakonas et al., 1995). Notch and Delta1 are expressed in the developing chick retina (Austin et al., 1995; Ahmad et al., 1997; Henrique et al., 1997; Silva et al., 2003; Nelson and Reh, 2009). We asked whether components of the Notch signaling pathway distinguish progenitor cells in preneurogenic and neurogenic zones of the developing retina. We examined expression of Delta1 using immunohistochemistry and in situ hybridization. As early as stage 16 (E2.5), Delta1 was expressed in different patterns in preneurogenic and neurogenic zones. In the central, neurogenic zone of the retina, Delta1 was expressed by isolated cells, many of which co-expressed a differentiation marker (Fig. 1*Ed, Gb*, 5*A*). The Delta1-positive cells in this

region were generally in the outer half of the retina (i.e. towards the mitotic layer), and fewer were present towards the inner retina where post-mitotic differentiating cells accumulate. Furthermore, the Delta1-positive cells in the neurogenic zone did not incorporate BrdU in a short pulse study (Fig. 6), and did not express proliferating cell markers including proliferating cell nuclear antigen (PCNA), phosphohistone H3 or Ki67 (not shown). M-phase cells also did not express Delta1 in this region (Fig. 5*Aa* arrows). These results suggest that in the neurogenic zone of the retina new post-mitotic cells express Delta1 in the mitotic layer and that they lose Delta1 expression as they migrate towards the inner retina. The progenitor cells in the neurogenic zone appeared not to express Delta1. This is consistent with previous descriptions of Delta1 expression in the developing retina and other regions of the central nervous system (Henrique et al., 1997).

In contrast to the neurogenic zone, Delta1 was strongly expressed by most if not all cells in the preneurogenic zone of the retina (Fig. 1*Ed, Fb*, 5*B*). The Delta1-positive cells in the preneurogenic zone incorporated BrdU in a 30 min pulse-labeling study (Fig. 6*B*), and co-expressed markers of cell division including PCNA, phosphohistone H3 or Ki67 (not shown). M-phase cells were also Delta1-positive in this region (Fig. 5*Ba* arrow). Reconstruction of entire retinas from serial sections at several ages showed that the spatial-temporal distribution of the Delta1-rich region matched that of the preneurogenic zone (Fig. 2). There was a gradual loss of Delta1 protein expression in an approximately 50 to 100 μm wide transition zone between preneurogenic and neurogenic zones (Fig. 1*Ed*, 5*C*). Delta1 immunoreactivity was undetectable in the cells just peripheral to the first differentiating cells, and re-appeared in post-mitotic neurons in the neurogenic zone.

The ciliary zone at the peripheral margin of the retina was devoid of Delta1 expression (Fig. 1*Ed*, 7).

It is worth noting that at the developmental stages we studied, more than 70% of the cells in the neurogenic zone of the retina were dividing progenitor cells. Thus, the absence of broad Delta1 expression in the neurogenic zone was not simply due to most cells having differentiated. Thus, these results show that Sox2/Delta1 expression identifies preneurogenic progenitor cells and distinguishes them from the Sox2-positive, Delta1-negative neurogenic progenitor cells (Fig. 1*F, G*). Although Delta1 expression by differentiating cells in the retina has been reported previously, the ubiquitous expression by progenitor cells in the preneurogenic zone of the retina has not been noted previously.

II.C.iv. E2A Expression Identifies Neurogenic Progenitor Cells

It would be expected that as progenitor cells mature from preneurogenic to neurogenic they up-regulate expression of proteins required to initiate neurogenesis, as well as down-regulate Delta1, which inhibits differentiation. Previous studies suggested that certain bHLH transcription factors promote neuronal differentiation (reviewed by Bertrand et al., 2002). We asked whether expression of any bHLH transcription factor is up-regulated during the transition from the preneurogenic to neurogenic state and whether any serve to identify neurogenic progenitor cells. We used in situ hybridization to examine expression of several factors including Ascl1, Neurod4, Atoh7, E2A, Neurod1 and Neurog2. Although all factors examined were expressed in the neurogenic zone of the developing retina, all except E2A were expressed only by a small subset of cells (data not shown), which is consistent with previous studies (Matter-Sadzinski et al., 2005; Trimarchi et al, 2008).

E2A was expressed by progenitor cells in the neurogenic zone of the developing retina. Our probe did not distinguish between the two splice variants encoded by the E2A gene, E12 and E47, so we use the gene name, E2A. The protein products of the

E2A gene promote neurogenesis by dimerizing with other proneural bHLH transcription factors (Johnson et al., 1992; Roberts et al., 1993; Shimizu et al., 1995; Fode et al., 2000). Sections of retina from stage 16 (E2.5) to stage 28 (E5.5) embryos processed for in situ hybridization with an E2A probe and for immunohistochemistry with the RA4 antibody showed that E2A and RA4 expression were co-extensive, although the two were not co-expressed in individual cells (Fig. 7). E2A expression was up-regulated in the transition zone between the preneurogenic and neurogenic zones, and its expression was reciprocal to that of Delta1. Direct comparison of Delta1 and E2A expression by double in situ hybridization showed that E2A expression is up-regulated as Delta1 expression is down-regulated and that the two are not co-expressed (Fig. 7). E2A expression was up-regulated just peripheral to the most peripherally located RA4-positive cell. E2A was expressed by the majority of progenitor cells in the neurogenic zone. Of the cells in the neurogenic zone that incorporated BrdU during a 30 minute pulse, 85% expressed E2A. Collectively, the results indicate that Sox2/E2A expression is a marker for neurogenic progenitor cells in the developing retina and that E2A expression is up-regulated as Delta1 expression is lost by progenitor cells during the preneurogenic to neurogenic transition. The difference in Delta1 and E2A expression makes a strong case for preneurogenic and neurogenic progenitor cells being fundamentally different, although they both express Sox2. The distinct transition zone in which Delta1 expression is lost and E2A expression comes up suggests that a highly coordinated mechanism controls this maturation step.

II.C.v. Interrupting Delta-Notch Signaling Converted Preneurogenic to Neurogenic Progenitor Cells

Since Delta1, a Notch ligand, is expressed by preneurogenic progenitor cells, we asked whether cell-cell interactions mediated by Notch have an essential role in maintenance of the preneurogenic state. As described previously, an oligonucleotide complementary to the lin-12/Notch repeat region of chick Notch1 mRNA was injected into one eye of embryos to knockdown Notch expression (Austin et al., 1995). An oligonucleotide with conservative rearrangements of the antisense sequence, the missense oligonucleotide, was injected into an eye of embryos to serve as a control. Eyes were injected with an oligonucleotide on E3.5 or E4.5, fixed 24 hours later and analyzed histologically. We observed several changes in the antisense treated retinas consistent with loss of the preneurogenic zone and expansion of the neurogenic zone. First, RA4-positive differentiating cells were present across a much greater extent of the retina, often up to the WFDC1-positive ciliary zone (Fig. 8*A, B*). Although some cells in the peripheral retina ceased further division and underwent premature differentiation following the antisense Notch treatment, the majority of the cells in this region continued to divide and to express the progenitor marker, Sox2 (Fig. 8*F*). Second, the Sox2-positive progenitor cells in the peripheral retina lost Delta1 expression (Fig. 8*Ab, E*). Third, the zone of E2A expression was expanded peripherally up to the border of the ciliary zone (Fig. 8*Ab, B*). These changes were seen in 45 out of 56 antisense Notch injected eyes. The preneurogenic zone was present as normal in all missense-treated, control retinas (28 out 28 missense-treated retinas; Fig. 8*C-D*). The expansion of the neurogenic zone in antisense Notch treated retinas was verified by measuring the length of the neurogenic zone (i.e. the region with RA4-positive cells) in sections of antisense treated and control retinas relative to the length of the entire retina in the sections. If antisense Notch converts preneurogenic to neurogenic progenitor cells, then the neurogenic zone in the antisense treated retinas would be larger relative to control retinas. Including two of the

nine embryos that showed only a partial phenotype, the neurogenic zone of the retina was increased 14% in sections of antisense treated retinas compared to the contralateral, untreated retinas (figure 8*I*; $n = 9$; $p < 0.001$). These results suggest that interrupting Delta-Notch signaling by knocking down Notch expression converts preneurogenic progenitors to neurogenic progenitors.

Several approaches in addition to the antisense Notch treatment were employed to verify the role of Notch signaling in the preneurogenic zone. First, an expression vector for a dominant-negative form of suppressor of hairless, Su(H)DBM, was electroporated into the peripheral retina of E3.5 embryos. Su(H)DBM expression has been shown to block Delta-Notch signaling (Wettstein et al., 1997). A GFP expression vector was co-electroporated to identify the transfected region of the retina. Thirty hours after electroporation, the retinas were analyzed for the distribution of differentiating cells. Three out of four eyes with Su(H)DBM electroporation showed premature neurogenesis in the peripheral retina (Fig. 8*G, H*). Similar to what we found with antisense Notch treatment, the differentiating cells were a minor population, and the majority of the cells in the transfected region continued to divide and to express Sox2. Transfection of the GFP vector alone did not induce premature neurogenesis (8 out of 8 eyes transfected with GFP vector alone). In other embryos, Notch signaling was interrupted in the developing retina by injecting an antisense Delta1 oligonucleotide or a γ-secretase inhibitor into the eye of E3.5 embryos. Twenty four hours later, we observed similar effects as those seen with antisense Notch treatment, which was scattered premature neurogenesis with persistent Sox2-positive, dividing cells in the peripheral retina (not shown). These observations are consistent with a scenario in which the resulting premature neurogenesis upon perturbation of Notch signaling was not merely due to a release of the cells from inhibition of neuronal differentiation, but rather to the conversion

of progenitor cells from preneurogenic to neurogenic. These results suggest that Delta-Notch signaling maintains the preneurogenic mode of division during normal retinal development and that interrupting Delta-Notch signaling converts preneurogenic to neurogenic progenitor cells.

II.C.vi. Shh Drives Maturation of Preneurogenic to Neurogenic Progenitor Cells

Delta1 expression is down-regulated as progenitor cells mature from preneurogenic to neurogenic, which would terminate Notch activation. The question remains as to what initiates down-regulation of Delta1. Previous studies suggested that a self-propagating wave of hedgehog signaling drives the progress of neurogenesis across the retina in vertebrates (Neumann and Nuesslein-Volhard, 2000; Stenkamp and Frey, 2003; Masai et al., 2005; Locker et al., 2006). We asked whether hedgehog signaling drives progenitor cell maturation in the developing chick retina. We first examined expression of a hedgehog family member, Sonic Hedgehog (Shh). Sections of early developing chick retina were processed for immunohistochemistry with an antibody to Shh and with the Brn3 antibody to identify the neurogenic front. Shh labeling was present in a subset of cells in the newly formed ganglion cell layer up to the neurogenic front (Fig. 9), a pattern similar to that reported previously for several vertebrate species (Jensen and Wallace, 1997; Neumann and Nuesslein-Volhard, 2000; Zhang and Yang, 2001). Thus, Shh is expressed in a spatiotemporal pattern consistent with it having a role in driving progenitor cell maturation.

We next asked whether misexpression of Shh in the preneurogenic zone would induce premature progenitor cell maturation. Shh was misexpressed in the preneurogenic zone by electroporation of a plasmid expression vector in the peripheral retina on E3.5. A GFP expression vector was co-electroporated. Embryos were allowed

to develop for an additional 30 hours. Premature neurogenesis as indicated by RA4 immunoreactivity took place in peripheral retina in the area of Shh misexpression (Fig. 10). However, only a subset of cells differentiated (Fig. 10*A, C, E, G*). The majority of cells in these regions remained as Sox2-positive progenitor cells (Fig. 10*G*). These progenitor cells, however, lost Delta1 expression (Fig. 10*C, E*). This loss of Delta1 expression by Sox2-positive cells is consistent with the suggestion that Shh initiated premature progenitor cell maturation.

II.D. Discussion

II.D.i. Molecular Distinction between Preneurogenic and Neurogenic Progenitor Cells

Progenitor cells in the early developing retina initially divide so that each cell division produces two cells that divide again. As development progresses, the mode of division changes so that a division can generate one or both cells that cease further division and differentiate. Previous work has not established whether the progenitor cells that exhibit these two modes of division are fundamentally different. We found that both progenitor cell types express Sox2. We initially distinguished neurogenic and preneurogenic zones of the developing retina by expression or absence of expression of differentiation markers. We show that virtually all progenitor cells in the preneurogenic zone expressed the Notch ligand, Delta1, and none of those in the neurogenic zone expressed Delta1. Conversely, virtually all progenitor cells in the neurogenic zone expressed high levels of the bHLH transcription factor, E2A, which was not seen in the preneurogenic zone. We suggest that Delta1 is a marker for preneurogenic progenitors, that E2A is a marker for neurogenic progenitors, and that these two progenitor cell stages are fundamentally different. Since all cells in a given region of the retina down-regulate Delta1 expression and up-regulate E2A expression prior to appearance of the first post-mitotic cell in that region, and since dividing cells do not appear to lose E2A expression or re-acquire Delta1 expression once neurogenesis has commenced, we suggest that the preneurogenic to neurogenic transition is a unidirectional maturation step made in unison by neighboring progenitor cells (Fig. 11).

It remains to be determined whether progenitor cells in other regions of the developing central nervous system display the same molecular distinction between

preneurogenic and neurogenic stages. Undifferentiated, proliferating cells in the caudal stem zone of spinal cord are characterized by uniform expression of Delta1 (Akai et al., 2005). The progenitor cells in this zone may be analogous to retinal preneurogenic progenitors. Unlike retina, however, blocking Notch signaling in this zone was not sufficient to induce neurogenesis. Also, it was reported that progenitor cells in neurogenic zones of spinal cord can express Delta1 (Hammerle and Tejedor, 2007), which may mean that spinal cord is fundamentally different than retina. Further work is needed to clarify the differences and similarities between spinal cord and retina.

Although the function of E12 and E47, the two proteins encoded by the E2A gene, has been linked to neurogenesis, our study describes for the first time that E2A expression is a common marker for neurogenic progenitor cells in the developing retina. Several lines of evidence suggest the possibility that E2A expression identifies neurogenic progenitor cells in other regions of the developing nervous system. First, the initiation of E2A expression appears to correlate with the first appearance of post-mitotic neurons in the brain (Ravanpay and Olson, 2008). Second, once neurogenesis commences, E2A expression is found in most cells in the proliferative layer of the neural tube (Roberts et al., 1993). Further analysis of E2A relative to preneurogenic and neurogenic zones of developing nervous system is needed to fully address this issue.

The number of progenitor cells continues to increase in areas of the retina with on-going neurogenesis (Dutting et al., 1983). This requires that neurogenic progenitor cells undergo at least some divisions that generate two progenitor cells. However, lineage tracing studies of neurogenic progenitors found mainly small clones (Wetts et al., 1989; Turner et al., 1990), which argues that these divisions are rare and that neurogenic progenitor cells undergo a limited number of divisions once neurogenesis commences.

II.D.ii. Maintenance of the Preneurogenic Mode of Division by Delta-Notch Signaling

Notch signaling is known to negatively regulate neuronal differentiation, and thereby maintain progenitor cells in the developing nervous system. Constitutive activation of Notch in the early developing retina blocked differentiation and caused all cells to continue to divide (Dorsky et al., 1995 and 1997; Henrique et al., 1997; Scheer et al., 2001). Conversely, blocking Notch activity resulted in premature differentiation and reduced cell proliferation (Jadhav et al., 2005; Yaron et al., 2006; Nelson et al., 2007). The view largely held is that without Notch activation progenitor cells cease further division and differentiate. We found, in contrast, that perturbation of Notch signaling in preneurogenic progenitor cells resulted in premature differentiation, but most cells continued to divide and to express Sox2. Furthermore, the cells that continued to divide up-regulated expression of E2A, a marker for neurogenic progenitors. Thus, reducing Notch activity appeared to convert preneurogenic progenitors to neurogenic progenitors. Another study concluded that Wnt signaling maintains the preneurogenic state in the developing retina (Kubo and Nakagawa, 2009). Further work is needed to clarify if or how these two signaling systems cooperate. We propose that Notch signaling in the preneurogenic zone of the retina normally maintains the preneurogenic mode of division. Interfering with Notch signaling causes preneurogenic progenitors to mature to neurogenic progenitors, thus leading to cell divisions that can produce cells that differentiate.

Notch signaling may maintain the preneurogenic state by repressing E2A. Our observation that inhibiting Notch signaling up-regulated E2A expression supports this view. The protein products of the E2A gene, E12 and E47, dimerize with proneural bHLH transcription factors such as Neurod1 and Neurogenin (Johnson et al., 1992; Roberts et

al., 1993; Shimizu et al., 1995; Fode et al., 2000). The proneural bHLH transcription factors lead to cell cycle withdrawal and neuronal differentiation (reviewed by Bertrand et al., 2002). E2A proteins stabilize the proneural bHLH transcription factors making the heterodimers more effective than proneural homodimers (Breslin et al., 2003; Vosper et al., 2007; Longo et al., 2008). E2A can be blocked by Notch signaling via Hes1 (Sasai et al., 1992; Bae et al., 2000). It is possible that up-regulation of E2A by reduction of Notch signaling bestows the neurogenic potential to progenitor cells.

II.D.iii. Control of Progenitor Cell Maturation

Preneurogenic progenitor cells switch their mode of division to neurogenic at a time in development determined by a cell's position in the retina. Previous work showed that Hedgehog (Hh) signaling is required for the normal advance of neurogenesis across the vertebrate retina (Neumann and Nuesslein-Volhard, 2000; Stenkamp and Frey, 2003; Masai et al., 2005; Locker et al., 2006) much as in the Drosophila eye (Heberlein et al., 1993; Dominguez and Hafen, 1997). We showed that Shh normally is expressed by a subset of newly differentiating ganglion cells in chick retina as reported previously (Jensen and Wallace, 1997; Neumann and Nuesslein-Volhard, 2000; Zhang and Yang, 2001) and that Shh misexpression can induce premature neurogenesis in regions that would normally have been preneurogenic. In regions where Shh was misexpressed, many cells down-regulated Delta1 expression but continued to express Sox2 and to divide. Based on these results we propose that preneurogenic progenitor cells near the neurogenic front are converted to neurogenic progenitor cells as Shh from nearby ganglion cells represses Delta1 expression, which would terminate Notch signaling.

Not all studies are consistent with Hh signaling driving the advance of the neurogenic front across the retina (Moshiri and Reh, 2004; Kay et al., 2005; Wang et al.,

2005). Clearly Hh signaling can be mitogenic in the developing retina (reviewed by Amato et al., 2004). Our results, however, suggest that Hh signaling reduces cell division by switching progenitor cells from the preneurogenic mode of division where the population grows exponentially to the neurogenic mode with more linear growth kinetics. Several factors could have led to these conflicting results. For example, Hh signaling may have different effects on preneurogenic and neurogenic progenitor cells. The mitogenic effect of Hh signaling may apply only to neurogenic progenitors. Many experimental approaches such as retinal dissociation used previously to study Hh signaling would have altered Delta-Notch signaling, which would confound the results. In addition, previous studies did not always distinguish between preneurogenic and neurogenic zones. Shh clearly has roles in neurogenic retina including determining cell fate (Zhang and Yang, 2001; Sakagami et al., 2009) that are independent of its role in promoting preneurogenic to neurogenic progenitor cell maturation. Further analysis is needed for complete understanding of the role of Hh signaling in the two progenitor cell populations.

Collectively our results show that retinal progenitor cells have fundamentally different preneurogenic and neurogenic stages, that the preneurogenic-to-neurogenic transition is a highly organized unidirectional step made in unison by neighboring cells, and that progenitor cell maturation is initiated by a non-cell autonomous mechanism. Showing that preneurogenic and neurogenic progenitor cells are fundamentally different provides an important insight into developmental mechanisms. Many investigators are working to identify the conditions that will convert stem cells to neural progenitor cells with the hope of using these cells for therapeutic cell replacement in the nervous system. Our findings suggest that these investigators need to consider another level of complexity, the distinction between preneurogenic and neurogenic progenitor cells.

Preneurogenic progenitor cells may be needed to obtain a sufficient quantity of cells, while neurogenic progenitor cells will be needed to generate functioning neurons.

Figure II-1. Distinct Preneurogenic and Neurogenic Zones in the Developing Chick Neural Retina The preneurogenic and neurogenic zones in the developing neural retina were defined by immunohistochemistry and in situ hybridization on sections of eyes at various developmental stages. In all panels, immunohistochemistry with the RA4 antibody (green) labels retinal ganglion cells, the first neuronal cell type generated during retinal development, and the presence of RA4-positive cells defines the neurogenic zone. Solid arrowheads indicate the most peripherally positioned differentiating cell, which we define as the neurogenic front. The neurogenic front divides the neurogenic zone (central) from the preneurogenic zone (peripheral). Open arrowheads indicate the approximate division between the ciliary zone (most peripheral) and the preneurogenic zone of the developing neural retina. DAPI staining (blue) labels all nuclei. 'L' indicates the developing lens. In micrographs **A**, **B** and ***C***, the nasal side of the retina is towards the left and the temporal side is towards the right. Micrographs ***D***-***E*** show the temporal half of the retina. ***A***-***D***, The Sox2 immunostaining labels neural progenitor cells in retina (red), as well as lens progenitor cells. At stage 14 (E2) just after formation of the optic cup, all cells in the future neural retina are Sox2-positive, preneurogenic progenitor cells. No cells are differentiating at this stage (i.e. no green cells) (***A***). At stage 16 (E2.5), a few cells in the center of the retina have begun to differentiate (green) (***B***). This is the first appearance of the neurogenic zone of the retina. Most of the retina is still preneurogenic at this stage (***B***). ***C-D***, As development progresses, the neurogenic front (closed arrowheads) advances towards the peripheral margin of the retina as the neurogenic zone in the center of the retina expands. ***Ea-d***, Micrographs show expression of WFDC1, Sox2, and Delta1 detected by fluorescent in situ hybridization in adjacent sections of a stage 23 (E4) retina. Cells in the peripheral margin of the retina, the ciliary zone, express WFDC1 ***(Eb)***, and are negative for Sox2 ***(Ec)***. Cells of the ciliary zone contribute to non-neural tissues of the eye including the ciliary body and iris. Delta1 is expressed by all cells in the preneurogenic zone and by isolated cells in the neurogenic zone of the retina (***Ed***). Delta1 is not expressed by cells in the ciliary zone (***Ed***). The dotted line indicates the transition zone between the preneurogenic and neurogenic zones. Micrographs ***F*** and ***G*** show in situ hybridization for Delta1 (red) and Sox2 (green) in two fields of the same section of a retina from a stage 25 embryo. Micrographs in a row (a-d) show the same field of view with different filter sets. ***F***, Progenitor cells in the preneurogenic zone of the retina express Delta1 and Sox2. Co-expression is indicated by yellow in the merged image (***Fd***). ***G***, Progenitor cells in the neurogenic zone express *Sox2* (green) but not Delta1 (red). Delta1 in the neurogenic zone is expressed by post-mitotic cells. Scale bars: ***A-E,*** 100 μm; ***F-G***,10 μm.

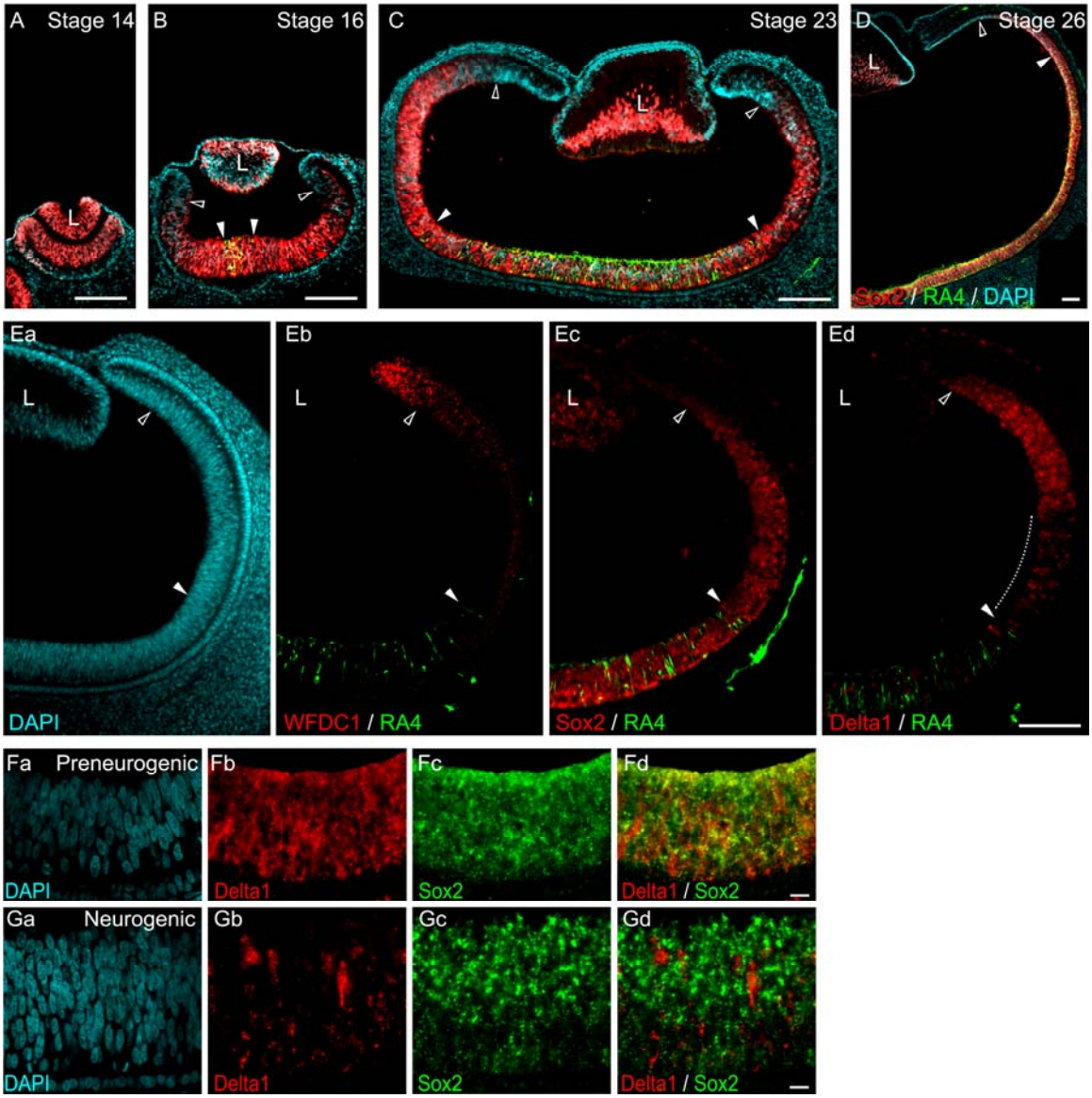

Figure II-1. Distinct Preneurogenic and Neurogenic Zones in the Developing Chick Neural Retina

Figure II-2. Delta1 Expression Relative to Cell Differentiation in Developing Retina These reconstructions of retinas show the distribution of Delta1 expression relative to differentiating cells. Serial sections of E4 and E5 retinas were processed for immunohistochemistry with an antibody to Delta1 and with the RA4 antibody. The overall length of the retina in each section was measured, as were the lengths and relative positions of the regions with Delta1 expression and with differentiating (RA4+) cells. These reconstructions were made by representing each section as a line. The portion of each section with all cells expressing Delta1 (i.e. preneurogenic retina) is represented by solid red in each line. The portion of each section with differentiating cells and with isolated Delta1-positive cells (i.e. neurogenic retina) is represented by solid green with red dots in each line. The optic fissure in the ventral retina is represented by the black line and was used to define the dorsal-ventral axis of the retina. At E4, the Delta1-rich, preneurogenic region encompassed most of the peripheral retina. By E5, the Delta1-rich region had separated into two areas, a smaller area at the lower temporal margin and a larger area covering most of the nasal and dorsal periphery.

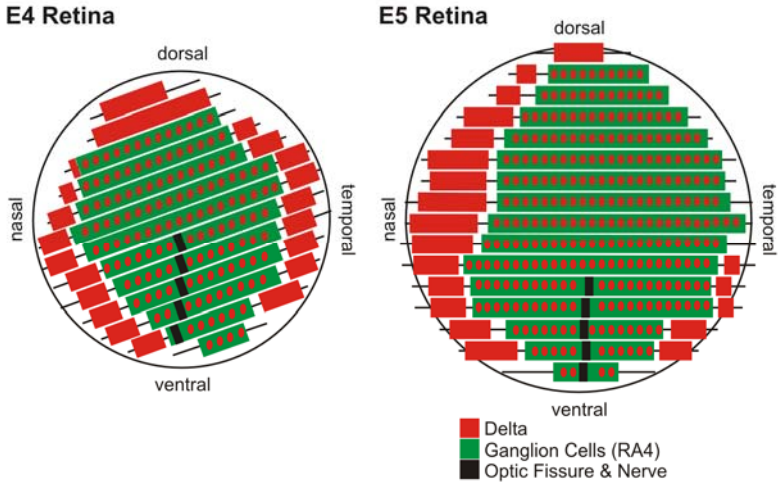

Figure II-2. Delta1 Expression Relative to Cell Differentiation in Developing Retina

Figure II-3. Differentiation of Dissociated Preneurogenic Progenitor Cells Cells isolated from preneurogenic retinas of E3.5 embryos were dissociated and cultured at low density. After 24 hours in culture, cells were fixed and processed for immunohistochemistry. The majority of isolated cells were positive for the differentiation marker, HuC/D (red). Typically few cells in larger clumps expressed a differentiation marker. DAPI staining shows all nuclei (blue). Scale bar: 10μm.

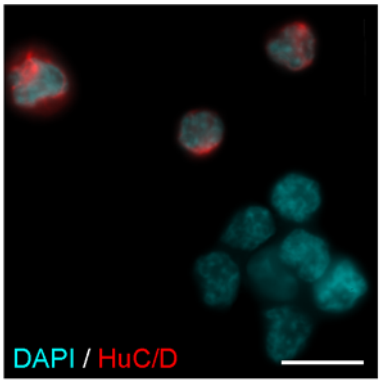

Figure II-3. Differentiation of Dissociated Preneurogenic Progenitor Cells

Figure II-4. Absence of Sox2 Expression by Differentiating Cells Micrographs showing expression of Sox2 (***A***), Isl1 (***B***), and NeuroD (***C***) detected by in situ hybridization. Micrographs show similar positions in adjacent sections of the neurogenic zone from a stage 30 retina. Sox2 expression was excluded from the Isl1-positive retinal ganglion cell layer (GCL) and the NeuroD-positive photoreceptor layer (ONL, outer nuclear layer). Scale bar: 50 μm

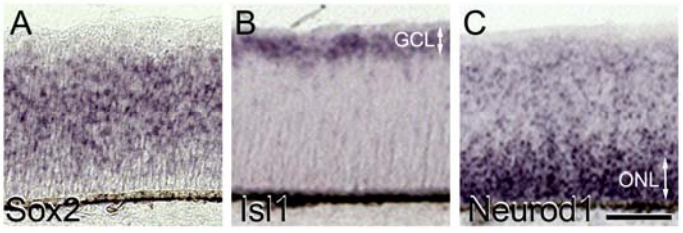

Figure II-4. Absence of Sox2 Expression by Differentiating Cells

Figure II-5. Delta1 Expression in Preneurogenic and Neurogenic Zones Fluorescence micrographs of chick retina show immunohistochemical staining with an antibody to Delta1 (red) and with the RA4 antibody (green), which shows ganglion cells, the first cell type to differentiate in the retina. Shown here is a stage 23 (E4) chick retina. DAPI staining (blue) shows all nuclei. The DAPI staining was omitted from the images on the right to better show the other markers. In each micrograph, the mitotic layer of the retina is towards the bottom, and the inner retina is towards the top. ***A***, In the central, neurogenic region of the developing retina where cell differentiation had begun as indicated by the presence of RA4-positive cells, only scattered isolated cells express Delta1. Dividing cells in M-phase (arrows) do not express Delta1 in this region of the retina. Newly post-mitotic, RA4-positive cells express Delta1 in or near the mitotic layer (asterisk). Delta1 expression is lost as the differentiating cells migrate towards the inner retina. ***B***, Delta1 is expressed by most, if not all, cells in the peripheral, preneurogenic zone. Dividing cells in M-phase are Delta1-positive (arrows). Differentiation has not commenced in this region as indicated by a lack of RA4-positive cells (i.e. no green cells). ***C***, Delta1 expression gradually decreases in the transition from the preneurogenic to neurogenic zones (dotted line). The transition zone is typically 50 to 100 μm wide. The solid arrowhead indicates the most peripherally positioned differentiating cell, the neurogenic front. Scale bars: ***A-B***, 10 μm; ***C***, 25 μm.

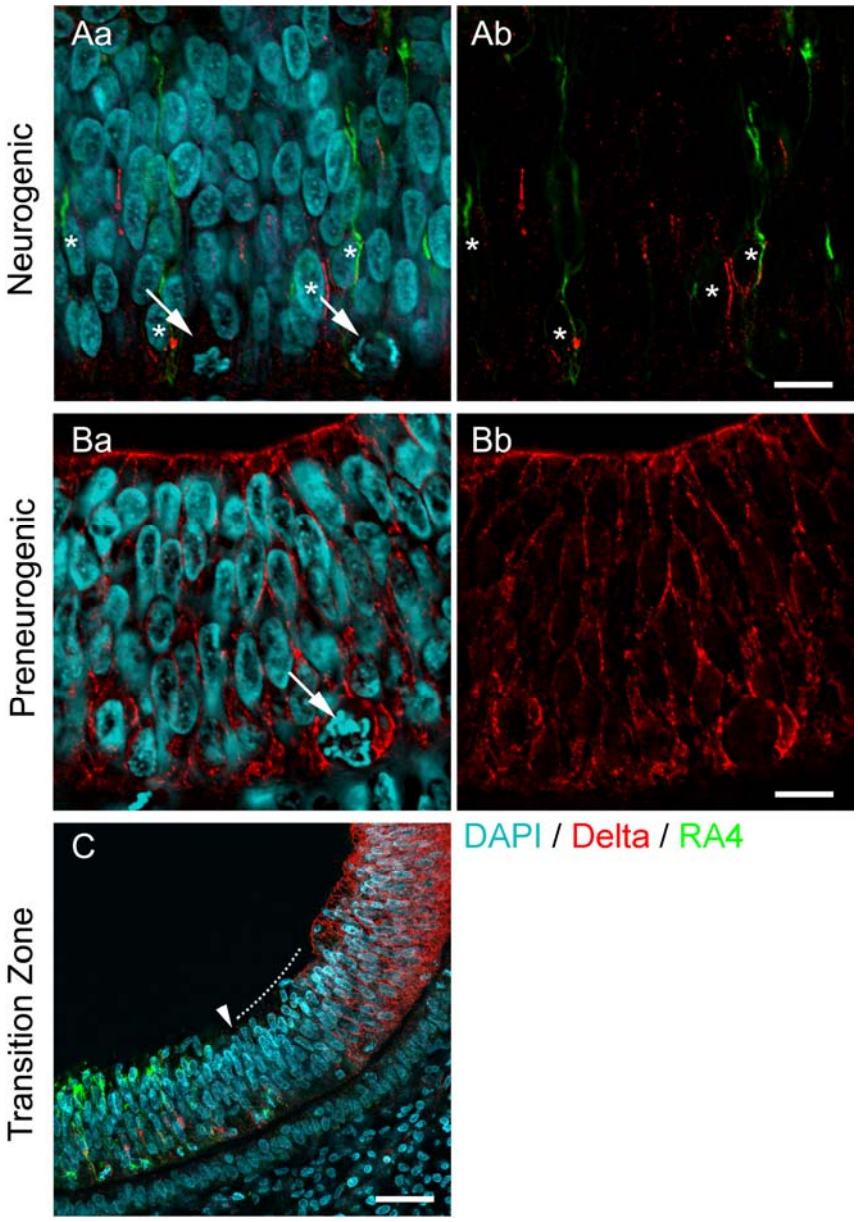

Figure II-5. Delta1 Expression in Preneurogenic and Neurogenic Zones

Figure II-6. Delta1 Expression Relative to Cell Division in Neurogenic and Preneurogenic Regions of the Retina Dividing cells were labeled by a BrdU injection into E4.5 chick embryos 30 min. prior to harvesting the eyes for histological analysis. Sections of the eyes were processed for in situ hybridization with antisense probe for Delta1 (red) and for immunohistochemistry with the RA4 antibody (blue) and an antibody against BrdU (green). ***A***, Micrograph of the neurogenic region of the developing retina shows that the scattered Delta1-expressing cells did not incorporate BrdU. ***B***, Micrograph of the preneurogenic region of the same retina shows that virtually all BrdU-positive cells express Delta1. Scale bar: 25μm. Both micrographs are at the same magnification.

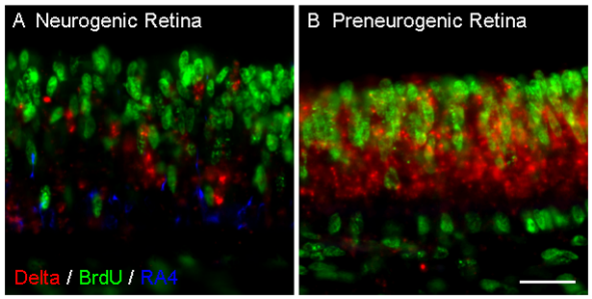

Figure II-6. Delta1 Expression Relative to Cell Division in Neurogenic and Preneurogenic Regions of the Retina

Figure II-7. E2A Expression in the Neurogenic Zone Double fluorescent in situ hybridization of chick retina shows the distribution of E2A (red) and Delta1 (green) expression (micrograph on the right). RA4 immunoreactivity (blue) identifies the neurogenic zone. The micrograph on the left shows DAPI staining of nuclei in the same section. Solid arrowheads indicate the most peripherally positioned differentiating cell, the neurogenic front. Open arrowheads indicate the approximate division between the ciliary and preneurogenic zones. Shown here is the temporal side of a section of stage 22 (E3.5) chick retina. E2A expression identifies neurogenic progenitor cells, while Delta1 expression identifies preneurogenic progenitor cells. As expression of Delta1 is down-regulated in the transition between preneurogenic and neurogenic zones, E2A expression is up-regulated. E2A is continuously expressed by all progenitor cells in the neurogenic zone. Scale bar: 100 μm

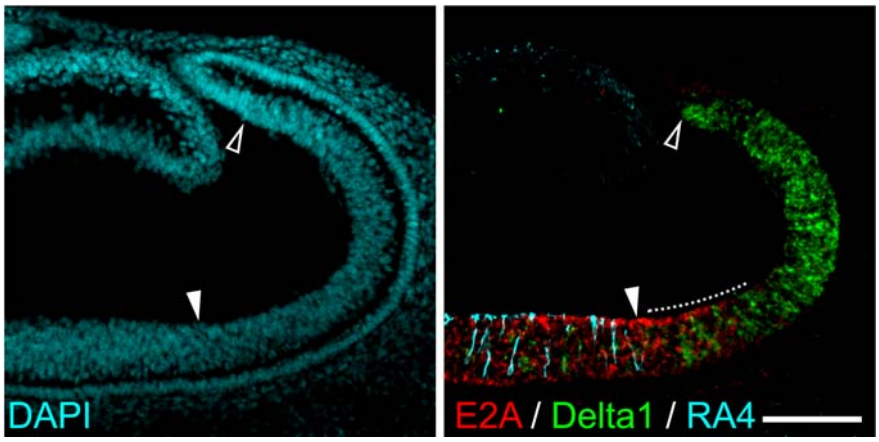

Figure II-7. E2A Expression in the Neurogenic Zone

Figure II-8. Conversion of Preneurogenic to Neurogenic Progenitor Cells Following Reduced Notch Signaling Notch signaling was reduced by treatment with (***A-B***) an antisense Notch oligonucleotide or (***G***) in ovo electroporation with a dominant negative form of Su(H) at E3.5. Experimental and contralateral control eyes were analyzed 24 hours later by in situ hybridization and immunohistochemistry. Immunostaining with the RA4 antibody identifies the neurogenic zone, and solid arrowheads indicate the most peripherally positioned differentiating cell, the neurogenic front. Open arrowheads indicate the approximate division between the ciliary and preneurogenic zones. DAPI staining shows all nuclei (blue). All micrographs show the temporal side of the retina. Micrographs ***Aa, Ca***, and ***Ea*** show of the identical sections and fields of view as micrographs ***Ab***, ***Cb***, and ***Eb***, respectively, but with different labels visible. ***A-B***, Cross sections of antisense treated or ***C-D***, contralateral control eyes were hybridized with probes to E2A, Delta1 and WFDC1. In antisense treated retinas, the neurogenic front was significantly more advanced towards the peripheral margin compared to the contralateral control retina. The neurogenic zone is shown by RA4 immunoreactivity (green) and E2A expression (red). The preneurogenic zone, identified by ubiquitous Delta1 expression, was absent following the antisense treatment. The ciliary zone identified by WFDC1 expression was unchanged in the antisense treated eyes. ***Ea-F***, In the region with premature neurogenesis induced by antisense Notch, Delta1 (detected by immunohistochemistry, green) was expressed predominantly by differentiating, RA4-positive cells (red). Delta1 was no longer expressed by progenitor cells (***Ea, Eb***). In this region, most cells, except newly differentiating cells (red), were still Sox2-positive progenitor cells (green) (***F***). The micrograph in ***F*** shows a neighboring section in the same retinal region as shown in ***E***. ***G-H***, Blocking Notch signaling in preneurogenic zone by in ovo electroporation with dominant negative Su(H) resulted in a similar preneurogenic to neurogenic conversion as seen following antisense Notch treatment. A GFP expression vector was co-electroporated to visualize transfected cells. The neurogenic front of the electroporated retina progressed further peripherally compared to the contralateral, control retina. ***I***, The expansion of the neurogenic zone following antisense Notch treatment was quantified. The length of neurogenic zone in cross sections, identified by the RA4 immunoreactivity, relative to the total length of retina was measured and compared between control and antisense Notch treated eyes ($n = 9$; $p<0.001$). Scale bars: ***A***-***D***, ***G***, ***H*** 100 μm; ***E***, ***F***, 10 μm. Micrographs in a row are at the same magnification.

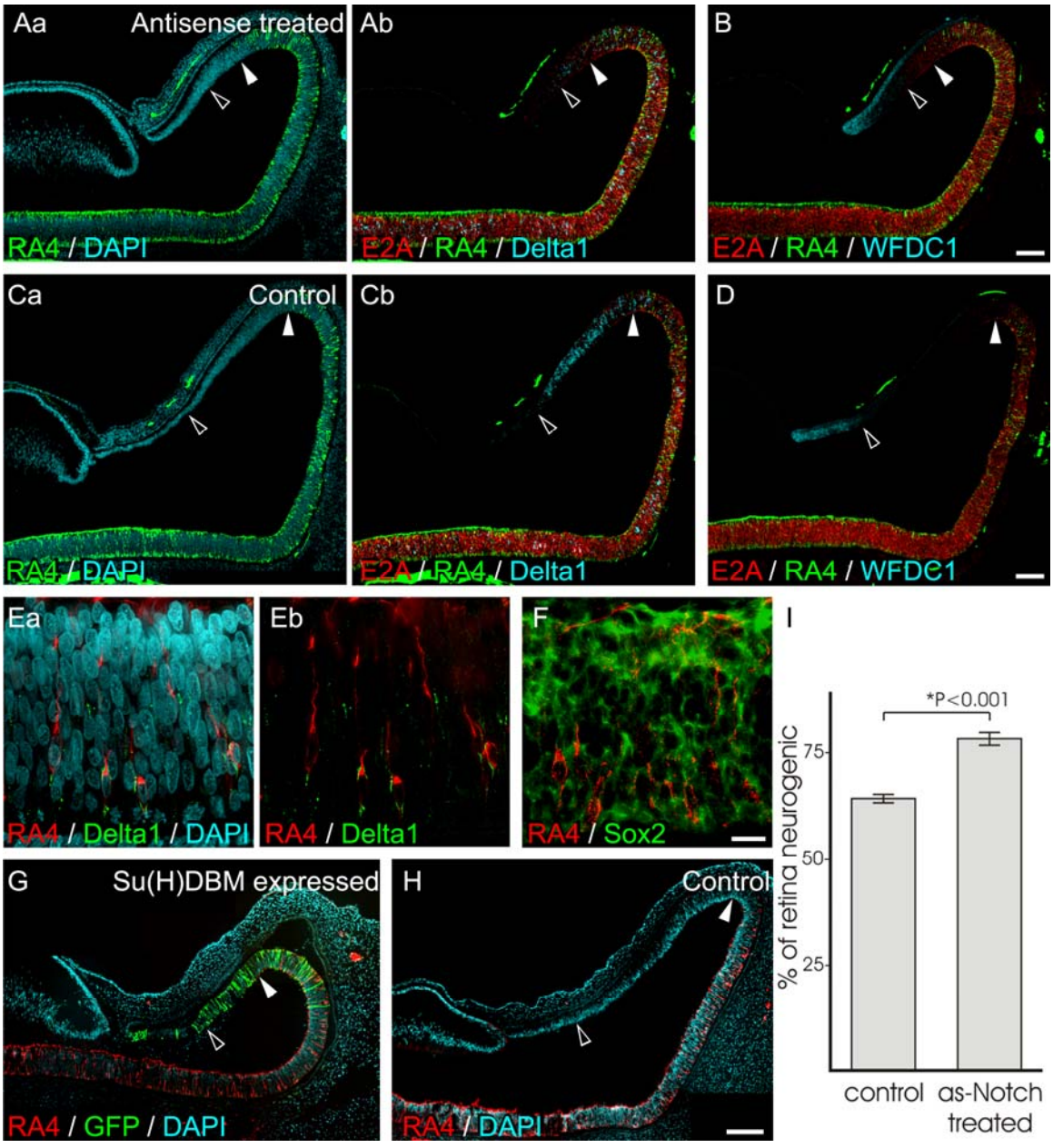

Figure II-8. Conversion of Preneurogenic to Neurogenic Progenitor Cells Following Reduced Notch Signaling

Figure II-9. Expression of Shh by a Subset of Newly Differentiating Ganglion Cells Shh expression (red) was examined in relation to the neurogenic front by immunohistochemistry on retinal sections from E4.5 embryos. An antibody to Brn3 (green) was used to identify ganglion cells. DAPI staining shows all nuclei (blue). A part of the retina including the neurogenic front is shown (dotted line) with the neurogenic domain on the left of the micrograph and preneurogenic domain on the right. Shh expression was detected in a subset of Brn3-positive ganglion cells up to the neurogenic front. Inset shows the boxed region near the neurogenic front at higher magnification. Shh expression could not be detected in the preneurogenic region, although secreted Shh presumably diffused into this region. Scale bar: 25μm.

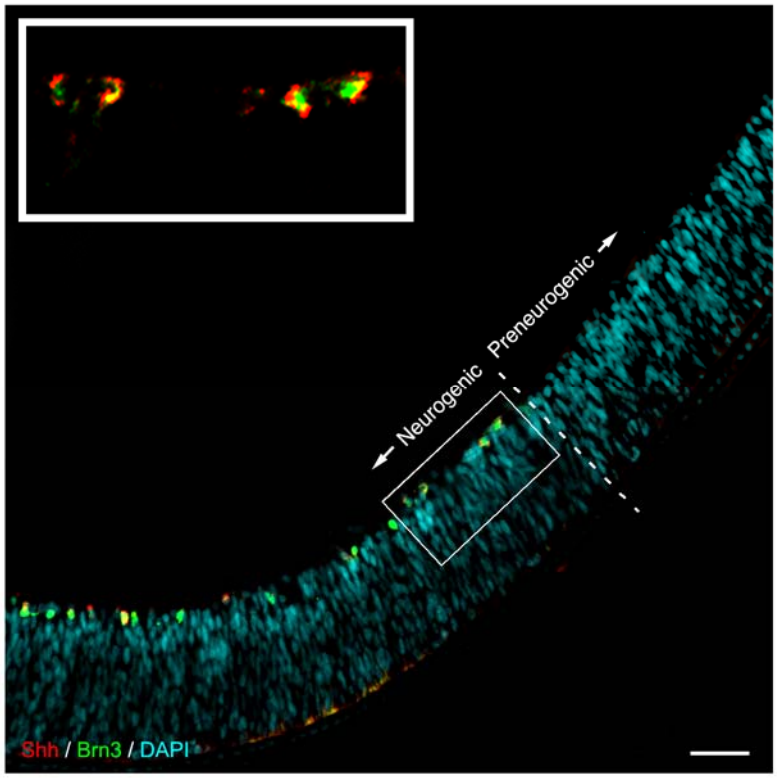

Figure II-9. Expression of Shh by a Subset of Newly Differentiating Ganglion Cells

Figure II-10. Progenitor Cell Maturation Induced by *Shh* Misexpression The preneurogenic zone of a retina was electroporated in ovo with a Shh expression vector together with a GFP expression vector. Thirty hours following electroporation the eyes were analyzed histologically. Micrographs show (left column) eyes with Shh misexpressed and (right column) the contralateral control eyes. Micrographs in a row are from the same embryo. The lower magnification micrographs in the experimental column were flipped in the horizontal axis so that the temporal side of the eye is to the right in all figures. ***A-B***, Micrographs show immunoreactivity for GFP (green, transfected cells) and RA4 (red, differentiating cells) and DAPI staining (blue, all nuclei). Open and closed arrowheads indicate the peripheral margins of the optic cups and the neurogenic fronts, respectively. The neurogenic front in regions of the retina with Shh misexpression had progressed further towards the peripheral margin compared to contralateral control retina. ***C-F***, Micrographs show immunoreactivity for Delta1 (green) and RA4 (red, differentiating cells) and DAPI staining (blue, all nuclei). Delta1 expression was significantly down-regulated in peripheral regions with ectopic Shh expression. Higher magnification images from the boxed regions in ***C***, ***D*** are shown in ***E***, ***F***, respectively. The boxes in the experimental and control retinas are at an equal distance from the peripheral margin on the temporal sides of the two retinas. ***E*** shows premature neurogenesis (i.e. RA4 expression) and downregulation of Delta1 expression in the retina in response to Shh misexpression. ***G-H***, Micrographs show immunoreactivity for Sox2 (green, neural progenitor), RA4 (red, differentiating cells) and DAPI staining (blue, all nuclei). Expression of Sox2 in the region with Shh misexpression indicates that most cells in the region of premature neurogenesis remain as progenitor cells. Scale bars: ***A, B, G, H,*** 100 μm; ***C, D***, 10 μm. Micrographs in a row are at the same magnification.

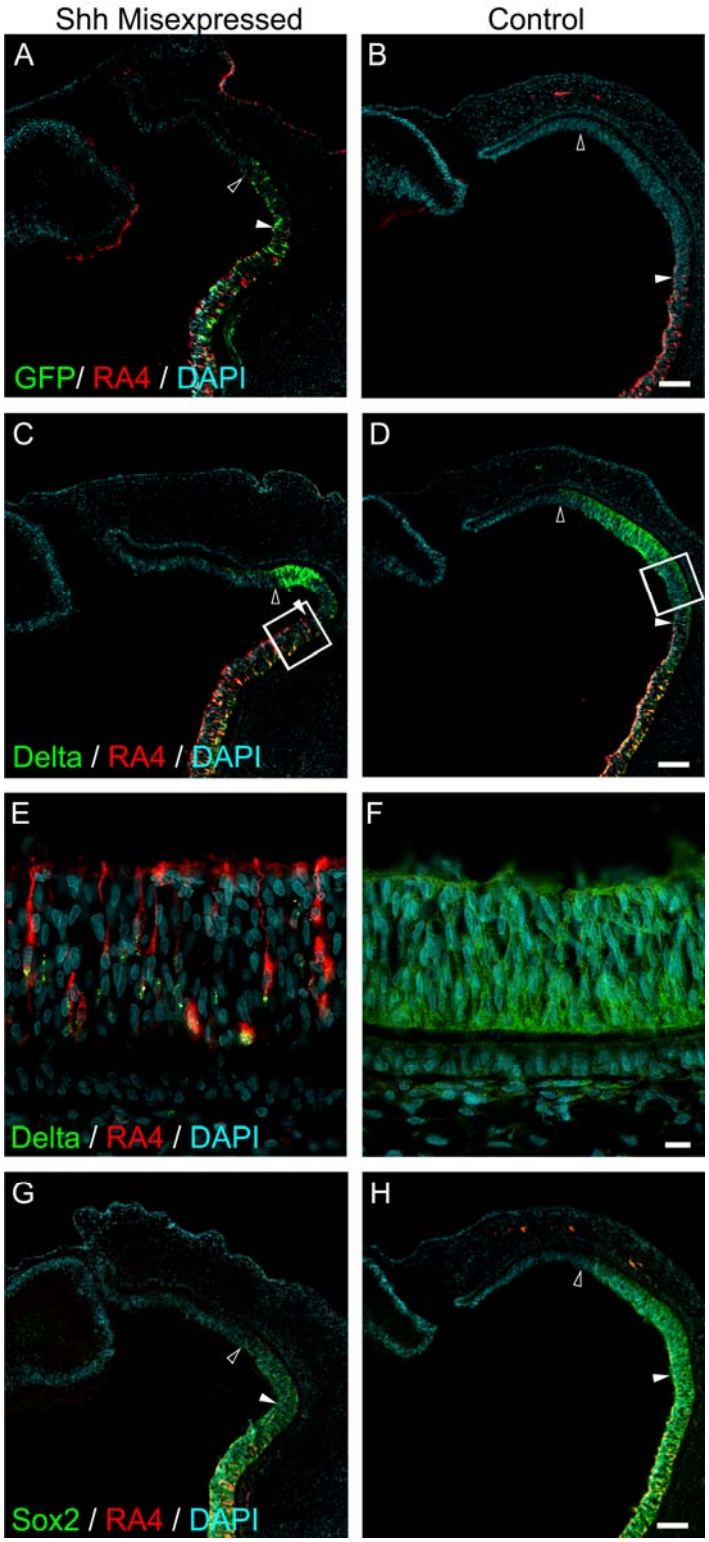

Figure II-10. Progenitor Cell Maturation Induced by Shh Misexpression

Figure II-11. Summary of Changes in Gene Expression during Retinal Progenitor Cell Maturation Early developing vertebrate neural retina consists of distinct zones that correspond to the consecutive steps of retinal cell maturation. These zones can be identified by expression of different markers. This chart summarizes the main zones of the developing retina with the bars representing expression of the molecular markers listed on the left side of the chart. Molecular markers were identified in this study by (·) protein expression, (.) RNA expression or (:) both. The red bars indicate genes expressed by dividing cells, as determined by co-labeling with a short pulse of BrdU, and the green bars indicate genes expressed by post-mitotic cells.

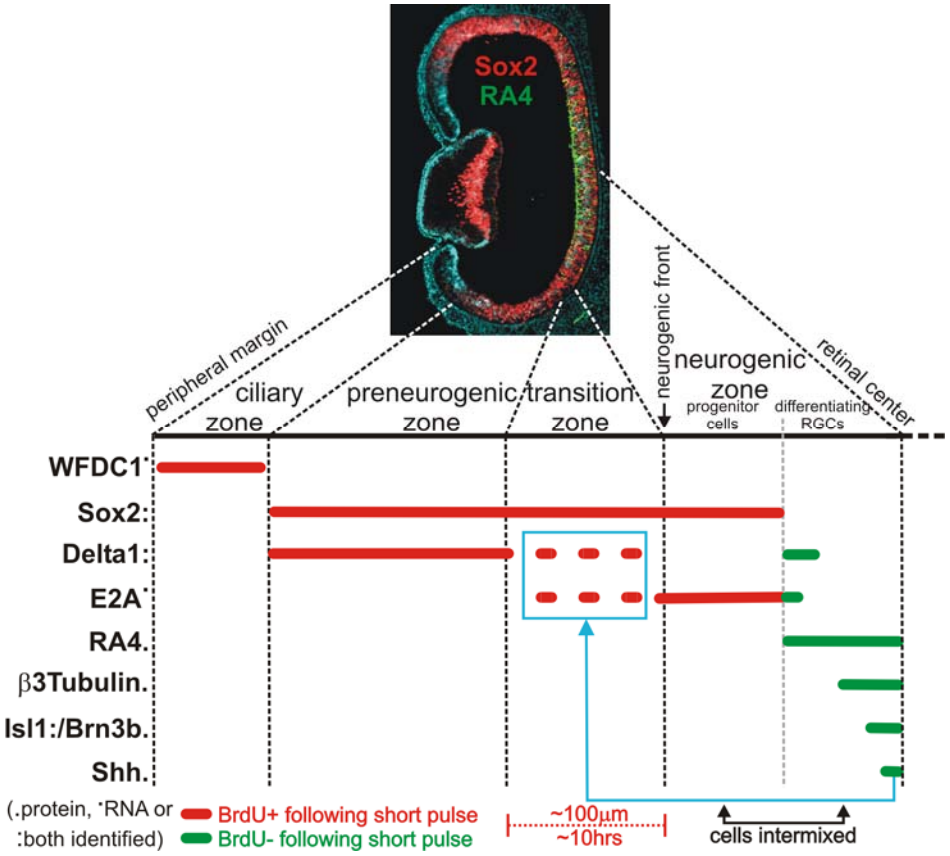

Figure II-11. Summary of Changes in Gene Expression during Retinal Progenitor Cell Maturation

CHAPTER III.

INITIATION OF NEUROGENESIS BY PRONEURAL bHLH TRANSCRIPTION FACTORS IN THE DEVELOPING VERTEBRATE RETINA

III.A. Introduction

The vertebrate neural retina is part of the central nervous system and is an attractive model to study the mechanism underlying neuronal development. The neural retina is composed of seven major cell types that arise during development from a common pool of progenitor cells (Turner and Cepko, 1987; Holt et al., 1988; Wetts et al., 1989; Turner et al., 1990). Although considerable progress has been made towards understanding many fundamental processes involved in neurogenesis, the molecular mechanisms that initiates neurogenesis in the retina is incompletely understood. Atonal (ato) is one of the proneural genes, which encodes members of the class II HLH family of transcription factors. It plays a key role in initiation of neurogenesis in the Drosophila eye imaginal disc. The uniform expression of ato in ectodermal cells just ahead of the morphogenetic furrow, the front of differentiation, specifies these cells as neuronal progenitor cells. The subsequent restriction of ato expression into evenly spaced single cells induces development of R8 photoreceptor cells (Jarman et al., 1994), the first neuron generated in each ommatidium.

Atoh7, a vertebrate orthologue of ato, is essential for normal development of ganglion cells, the first neuron generated in the vertebrate retina (Rubinson and Cain, 1989; Snow and Robson, 1994; Belecky-Adams et al., 1996). Genetic deletion of Atoh7 gene resulted in a severe loss of ganglion cells (Brown et al., 2001; Liu et al., 2001). Atoh7 promotes expression of other genes involved in ganglion cell differentiation (Hutcheson and Vetter, 2001; Liu et al., 2001). It seemed likely that Atoh7 initiates the neurogenic program that leads to ganglion cell differentiation. Several lines of evidence, however, suggest otherwise. Misexpression of Atoh7 in the developing retina failed to initiate neurogenesis efficiently (Liu et al., 2001). Furthermore, Atoh7 is thought to be

expressed after cells have withdrawn from the mitotic cycle and begun to differentiate (Yang et al., 2003). This would suggest that another factor or factors act upstream of Atoh7 initiates the differentiation program.

Previous studies identified multiple proneural genes expressed in the developing retina. These include Ascl1, Neurod1, Neurod4, Neurog2 (Guillemot and Joyner, 1993; Gradwohl et al., 1996; Jasoni and Reh, 1996; Sommer et al., 1996; Acharya et al., 1997; Kanekar et al., 1997; Roztocil et al., 1997; Takebayashi et al., 1997; Korzh et al., 1998; Brown et al., 1998). Their relatively early onset of expression in the retina suggests their potential role in initiation of neurogenesis (Brown et al., 1998; Matter-Sadzinski et al., 2001). Consistent with this, when ectopically expressed in the retinal pigmented epithelial cells cultured in vitro, Neurog2 or Neurod1 (Yan and Wang, 2000a, 2000b; Yan et al., 2001), was sufficient to generate multiple retinal cell types. Although considerable progress has been made towards understanding the role of these genes in determining cell fate (reviewed in Hatakeyama and Kageyama, 2004; Ohsawa and Kageyama, 2008), the role of proneural genes in initiation of neurogenesis in the retina has not been fully tested.

The developing retina exhibits a central-peripheral gradient of maturation with the center more mature and the periphery less mature. In the developing chick retina, a neurogenic front is present for a period of several days that progressively advances from the center towards the periphery of the retina (Dutting et al., 1983; Prada et al., 1991; McCabe et al., 1999; Yang et al., 2009). The ganglion cell is the first postmitotic cell type generated at the neurogenic front. We reasoned that the genes that initiate ganglion cell genesis would be expressed just peripheral to the neurogenic front and that their sequence of expression would be mapped in their patterns of expression. We used multi-probe fluorescence in situ hybridization to compare the patterns of expression of

each of the proneural genes to one-another based on their relative central-peripheral positions peripheral to the neurogenic front. Here we show that expression of a number of proneural bHLH factors comes up in a stereotypic temporal sequence prior to the generation of ganglion cells. Ascl1 and Neurog2 are the first genes expressed. Individual progenitor cells express heterogeneous combinations of proneural genes prior to the onset of ganglion cell genesis. These were followed by expression of Neurod1 and Neurod4, and finally Atoh7 was expressed just prior to terminal mitosis. Misexpression of Ascl1 or Neurog2 in preneurogenic retina was sufficient to initiate ganglion cell genesis. Misexpression of Neurog2 initiated the stereotypic sequence of proneural gene expression that normally precedes ganglion cell genesis. Although Ascl1 expression was sufficient to initiate ganglion cell genesis, it functioned by a mechanism distinct from that of Neurog2.

III.B. Experimental Procedures

Animals and Tissue Preparation

Pathogen-free, fertilized While Leghorn chicken eggs were obtained from Hy-line (Spencer, Iowa). Eggs were incubated at 37°C, 98% relative humidity. Embryos were staged according to Hamburger and Hamilton (1951). Once embryos reached the desired developmental stage for characterization, embryos were removed from the eggs and prepared in one of three ways as follows. To prepare cryosections, the harvested embryos were fixed in 4% paraformaldehyde/ 0.1M phosphate buffer, pH 7.4, for 1.5 hours, cryoprotected in 20% sucrose/ 0.1M phosphate buffer, pH 7.4, overnight at 4°C, and then embedded in O.C.T compound (from Electron Microscopy Sciences, Hatfield). Twenty micron thick frozen sections were prepared. To obtain retinal flat mounts, retinas were dissected from the embryos, fixed for 1 hour. Procedures such as immunohistochemistry were performed on the fixed retinas, and then the retinas were mounted flat on glass slides by making four cuts around the periphery. For quantitative analysis, slides with dissociated central retinal cells were prepared. Retinas were first dissected, and the central regions were collected. Cells were dissociated by incubation in 0.05% Trypsin/0.53mM EDTA for 15 minutes at 37°C and then triturated using pulled glass pipettes. The dissociated cells were plated and dried onto glass slides, and fixed for 10 minutes. Superfrost Plus glass slides (from Fisher Scientific, Pittsburgh) were used for all tissue preparations that were subject to in situ hybridization.

Double Fluorescent In Situ Hybridization and Immunohistochemistry

To examine expression of multiple genes simultaneously, tissue sections or dissociated cell preparations were processed for double fluorescent in situ hybridization and subsequent immunohistochemistry. For double fluorescence situ hybridization, antisense

RNA probes to the genes of interest were synthesized and labeled either with Fluorecein-12-UTP (from Roche, Indianapolis) or with Digoxigenin-11-UTP (from Roche, Indianapolis) by in vitro transcription. Prior to hybridization with RNA probes, tissue preparations were re-hydrated in Phosphate buffered saline (PBS), post-fixed in 4% paraformaldehyde/ PBS for 10 minutes, and quenched of endogenous peroxidase activity by treatment with 3% H_2O_2 for 10 minutes. Subsequently, sections were treated with 3µg/ml proteinase K followed by 5 minute fixation in 4% paraformaldehyde/ PBS, acetylated in triethanolamine-HCl for 10 minutes, permeabilized in 1% Triton X-100/ PBS, and prehybridized in hybridization solution for 2 hours. Pretreated tissue preparations were hybridized simultaneously with fluorescein- and digoxigenin-labeled RNA probes for 14 hours at 65°C, followed by two 30 minute washes in 50% formamide/ 1x SSC at 65°C and another two 30 minute washes in TBS/ 0.1% Tween-20 at room temperature. Fluorescein-labeled probes was detected first with anti-fluorescein antibody conjugated with alkaline phosphatase (from Roche, Indianapolis). TBS/ 0.1% Tween-20/ 2% blocking reagent/ 20% heat-inactivated sheep serum was used to block non-specific binding of the antibody and TBS/ 0.1% Tween-20/ 2% blocking reagent/ 1% heat-inactivated sheep serum was used as a carrier solution. Subsequently, tissue was incubated in Fast Red substrate (from Roche, Indianapolis) dissolved in 0.1M Tris, pH8.2, until the signal reached the desired intensity, which typically took 1-4 hours. Tissue preparations were washed and blocked in 0.5% Blocking reagent (from Roche, Indianapolis)/ TBS. Digoxigenin-labeled probes were detected by anti-digoxigenin antibody conjugated with peroxidase (from Roche, Indianapolis) diluted in 0.5% Blocking reagent/ TBS and developed using TSA™ Plus Fluorescein System (from Perkin-Elmer, Waltham). After double fluorescence in situ hybridization, tissues were processed for immunohistochemistry as described previously (Waid and McLoon, 1995). cDNAs in

plasmid vectors were used as templates to synthesize the RNA probes. The following cDNAs were used: Ascl1, Atoh7, Delta1 (a gift from David Ish-Horowicz, Oxford University), E2A (a gift from Klemens Meyer, University of Cambridge), Isl1 (a gift from Sam Pfaff, Salk Institute), Neurod1 (a gift from Elise Lamar, Salk Institute), Neurod4, Neurog2. Antibodies used for immunohistochemistry includee RA4 antibody (McLoon and Barnes, 1989), anti-Myc, anti-HA and anti-BrdU.

In Ovo Electroporation

To induce ectopic expression of Neurog2 or Ascl1 in vivo, in ovo electroporation was used as described previously (Muramatsu et al., 1996; 1997). The vitelline membrane was removed from E3.5 embryos, and approximately 300ng of an expression vector was microinjected into the vitreous of the right eye. Three pulses of electric current were applied using platinum electrodes placed on either sides of the injected eye at 25V for 50 msec each with 950msec between pulses. Electroporated embryos were incubated at 37°C with 5% CO_2 for 24 hours until harvested at E4.5. In misexpression studies with post electroporation times shorter than 24 hours, in ovo electroporation was done at an appropriate age between E3.5 and E4.5 so that electroporated embryos were also harvested at E4.5. The expression vectors used included pCS2-cAscl1 and pCS2-cNeurog2-HA.

Microscopy

Fluorescence signals were viewed with a Leica DMR fluorescence microscope. Digital images were captured from the microscope with a Photometrics Quantix camera. For some high power micrographs, images were taken at multiple planes through the thickness of tissue sections and deconvolved using Microtome (VayTek) within the Image-Pro Plus image-processing program (Media Cybernetics). Some high power

micrographs were also obtained with an Olympus FluoView™ FV1000 confocal microscope.

III.C. Results

III.C.i. Expression of Multiple Proneural Genes Precedes the Generation of Retinal Ganglion Cells

We first asked which proneural bHLH transcription factors are expressed prior to the onset of ganglion cell genesis in the retina. The developing retina exhibits a central-peripheral gradient of maturation from the more mature center to the less mature periphery. As the ganglion cell is the first neuron generated in the retina (Rubinson and Cain, 1989; Snow and Robson, 1994; Belecky-Adams et al., 1996), the most peripherally positioned ganglion cell in a section indicates the position of the neurogenic front. The genes required to initiate ganglion cell genesis would be expressed just peripheral to the neurogenic front. We used in situ hybridization on horizontal sections through the center of embryonic chick retina to examine the pattern of expression of proneural genes. The genes examined included Ascl1, Atoh7, Neurod1, Neurod4 and Neurog2. We used the immunohistochemistry with RA4 antibody to identify differentiating ganglion cells and to determine the position of the neurogenic front. The RA4 antigen is a microtubule associated protein expressed very early in ganglion cell differentiation (McLoon and Barnes, 1989; Waid and McLoon, 1995). Neuronal tubulin (nTub), another marker for newly differentiating ganglion cells (Snow and Robson, 1994, 1995), was used to identify the position of the neurogenic front in place of RA4 in some cases. It is worth noting that the most peripheral nTub positive cell is slightly more centrally positioned than the most peripheral RA4 positive cell (Fig. 3*A*, 3*B*), reflecting the slightly later onset of nTub expression compared to RA4 expression. Individual tissue sections were processed both for proneural gene in situ hybridization and RA4 immunohistochemistry. In the developing chick retina, neurogenesis begins in the center of the retina at embryonic day

2 (E2; HH stage 16) (Yang et al., 2009). The neurogenic front moves progressively more peripheral until E8 (HH stage 34), at which time cells are differentiating across the entire retina and a neurogenic front is no longer present. Thus, our analyses focused on E3-E8 retina. We invariably found that all five of the proneural genes examined were expressed in the developing retina peripheral to the neurogenic front throughout this period of development (Fig. 1*B-G* and not shown). All five proneural genes were expressed within approximately 100μm of the neurogenic front. This indicates that expression of Ascl1, Atoh7, Neurod1, Neurod4 and Neurog2 precedes ganglion cell differentiation. This suggests the possibility that one or more of these factors initiates the neurogenic program leading to ganglion cell genesis in the developing retina.

III.C.ii. Cells Expressing a Variety of Proneural Genes Differentiate as Retinal Ganglion Cells

Expression of Atoh7 is required for ganglion cell differentiation (Brown et al., 2001; Liu et al., 2001). Likewise, expression of other proneural genes has been linked to development of other retinal cell types (reviewed in Hatakeyama et al., 2001; Hatakeyama and Kageyama, 2004). Thus, it is not clear whether progenitor cells expressing proneural genes other than Atoh7 generate ganglion cells. We asked whether any of the proneural genes are expressed by early differentiating retinal ganglion cells. We looked for co-expression of each of the proneural genes and the ganglion cell marker, RA4, on sections of E4.5 (stage 26) chick retina. Cells begin to express ganglion cell markers including the RA4 antigen within minutes of undergoing their terminal mitosis (Waid and McLoon, 1995). The newly differentiating ganglion cells then migrate to the inner retina, where they accumulate in the ganglion cell layer. We found that all five proneural genes examined, Ascl1, Atoh7, Neurod1, Neurod4, and

Neurog2, were expressed in at least a subset of newly differentiating ganglion cells (Fig. 2*A*-*E* asterisks). Individual RA4 positive cells in or near the mitotic layer did not express all of these genes (Fig. 2*A*-*E*). The degree of co-expression, however, varied among the proneural genes examined. These results show that multiple proneural genes are expressed by differentiating ganglion cells. This suggests that progenitor cells expressing Ascl1, Neurog2, Neurod1 and/or Neurod4 can generate ganglion cells. These findings also suggest that one or more of these factors may be essential for initiating ganglion cell differentiation.

III.C.iii. The Onset of Proneural Gene Expression Prior to Ganglion Cell Genesis Is in a Stereotypic Sequence

We next asked whether there are temporal differences in the onset of expression of the various proneural genes prior to ganglion cell genesis. We compared expression of two proneural genes at a time by multi-probe fluorescence in situ hybridization followed by immunohistochemistry with the RA4 antibody. The relative time difference between the onset of expression of any two proneural genes was reflected in the position of the most peripherally located cell expressing each gene. One gene expressed by cells more peripherally positioned than a second gene would indicate that expression of the first gene comes up earlier than the second gene. Ascl1 and Neurog2 were always expressed more peripherally than the other factors indicating that they are the first proneural genes expressed before ganglion cell genesis. The peripheral boundary of the Ascl1 expression domain was slightly peripheral to that of the Neurog2 (Fig. 3*A*). In stage 26 retinas, approximately 30µm separated the peripheral boundaries of the Ascl1 and Neurog2 expression domains. This was found consistently in all retinas and at all stages examined. This indicates that Ascl1 expression comes up in progenitor cells

earlier than does Neurog2 expression and that generation of postmitotic neurons follows the expression of both genes. Direct comparison of expression of other pairs of proneural genes revealed a consistent temporal sequence in their expression. Ascl1 and Neurog2 expression began earlier (i.e. more peripheral) than the other proneural genes examined including Neurod4, Neurod1, and Atoh7 (Fig. 3*B-D*). The peripheral boundaries of Neurod1 and Neurod4 expression appeared to be the same with neither consistently more peripheral than the other (Fig. 3*C*). This indicates that expression of Neurod1 and Neurod4 begins at approximately the same time. However, expression of both Neurod1 and Neurod4 precedes the neurogenic front (Fig. 1*E*, *F*, 3*C*). The most peripheral extent of Atoh7 expression was just slightly peripheral to that of RA4 expression (Fig. 3*D*). Also, Atoh7 expression consistently came up central to the onset of expression of the other proneural genes examined (Fig. 3*D*), indicating that it is the last of these factors to be expressed. This temporal sequence of proneural gene expression was found consistently in retinas from all developmental stages examined. These findings indicate that expression of the proneural bHLH transcription factors come up in a stereotypic sequence just prior to the onset of ganglion cell genesis:

Ascl1 > Neurog2 > Neurod1 and Neurod4 > Atoh7

Previously we showed that progenitor cells undergo maturation from a preneurogenic state to a neurogenic state prior to generation of postmitotic cells in the retina (Yang et al., 2009). Preneurogenic progenitor cells divide so that each division produces two cells that divide again. Preneurogenic progenitors express Sox2 and the Notch ligand, Delta1. During maturation to the neurogenic state, preneurogenic progenitor cells lose Delta1 expression as they approach the neurogenic front. We asked what the temporal relationship is between the onset of proneural gene expression and progenitor cell maturation. Expression of Ascl1, the first proneural factor expressed,

came up just central to the region in which Delta1 expression began to be down-regulated (Fig. 1*B*). The peripheral boundaries of expression of the other proneural genes, those expressed after Ascl1, also were central to the region in which Delta1 expression began to be down-regulated. This is consistent with previous studies showing that Notch activation directly represses proneural gene expression (Nishimura et al., 1998; Kageyama et al., 2007). These results suggest that the onset of proneural gene expression occurs immediately following progenitor cell maturation from preneurogenic to neurogenic.

III.C.iv. Retinal Progenitor Cells Are Heterogeneous Prior to Neurogenesis

We asked whether individual progenitor cells express the same set of proneural genes at the onset of ganglion cell differentiation. To address this, we examined expression of each proneural gene in newly differentiating ganglion cells. Cells were dissociated from the neurogenic zone of stage 26 chick retinas and plated on glass slides. The plated cells were then processed for in situ hybridization for various proneural genes and for RA4 immunohistochemistry. The RA4 antigen is present in the cell body of newly differentiating ganglion cells immediately following terminal mitosis (Waid and McLoon, 1995). As ganglion cells migrate from the mitotic layer, the RA4 antigen become restricted to the leading process and then to the axon. Since all processes are lost during dissociation, the RA4 antibody can detect only newly postmitotic ganglion cells. Atoh7 was detectable in 91% of RA4-positive cells (Fig. 5*A*). This is consistent with the well established function of Atoh7 in ganglion cell development. It is worth noting, however, that Atoh7 was not detected in 9% of newly formed ganglion cells (Fig. 5*A*). Each of the other proneural genes studied, Ascl1, Neurog2, Neurod1 and Neurod4, was expressed only by a subset of the newly differentiating ganglion cells (Fig. 5*A*). The

percentage of RA4-positive cells expressing each gene varied. These data suggest that each proneural gene is expressed by a subset of newly differentiating ganglion cells.

We directly compared expression of pairs of proneural genes to further address the diversity among progenitor cells that are in preparation of ganglion cell genesis. Expression of Atoh7 is the last among the proneural genes examined, and its expression is followed immediately by terminal division and ganglion cell differentiation. If individual progenitor cells express all five proneural genes before generation of ganglion cells, then all Atoh7-positive progenitor cells will express the other proneural genes. If individual progenitor cells express different combinations of proneural genes, then at least some Atoh7-positive cells will not express some subset of the other proneural gene. To distinguish between these two possibilities, we looked for co-expression of each proneural gene with Atoh7 using multi-probe fluorescence in situ hybridization on cross sections of stage 26 chick retina. To limit analysis to the progenitor cells and to avoid postmitotic cells, we examined expression of pairs of proneural genes in the transition zone, just peripheral to the neurogenic front. RA4 immunoreactivity was used to identify the position of the neurogenic front. Individual Atoh7-positive cells expressed varied proneural genes. Atoh7-positive cells were found both with and without Neurod1 expression (Fig. 4*Aa*, *Ab,* yellow and red asterisks, respectively). The degree of co-expression was quantified using cells dissociated from the neurogenic zone of stage 26 chick retinas. Forty three percent of Atoh7-positive cells had detectable Neurod1 expression (Fig. 5*B*). There also was a population of cells that expressed Neurod1 and not Atoh7 (Fig. 4*Aa*, *Ab*, green asterisks). Similarly, Atoh7-positive cells were found with and without Neurod4 expression (Fig. 4*Ba*, *Bb,* yellow and red asterisks, respectively, 5*B*). Some cells that expressed Neurod4 were negative for Atoh7 expression. Expression of Neurod1 and Neurod4 is found in overlapping, yet distinct populations of

progenitor cells although Neurod1 and Neurod4 are expressed at about the same time during the normal retinal development. Some progenitor cells expressed either gene only (Fig. 4*Ca*, *Cb,* green or red asterisks), whereas some progenitor cells expressed both genes (Fig. 4*Ca*, *Cb,* yellow asterisks). Atoh7 expression was also compared to expression of Ascl1 or Neurog2. Only subset of Atoh7-positive cells co-expressed Neurog2 (Fig. 4*Ea*, *Eb,* yellow asterisks, 5*B*). In contrast to Neurod1, Neurod4, and Neurog2, Ascl1 was not expressed by Atoh7-positive cells (Fig. 4*Da*, *Db,* red asterisks, 5*B*). Taken together, these data suggest that individual progenitor cells in the transition zone express heterogeneous combinations of proneural genes.

III.C.v. Misexpression of Ascl1 or Neurog2 Can Initiate Premature Neurogenesis

The early expression of Ascl1 and Neurog2 and the presence of these factors in newly differentiating ganglion cells suggest that one or both of these factors may initiate proneural gene expression that leads to the ganglion cell genesis. To test this, we asked whether misexpression of Ascl1 or Neurog2 in preneurogenic retina was sufficient to initiate ganglion cell differentiation. Using in ovo electroporation, we misexpressed Ascl1 or Neurog2 in the periphery of E3.5 retina, the preneurogenic zone at this stage. Embryos were fixed 24 hrs after transfection. Sections or whole mounts of the transfected retinas were processed for immunohistochemistry or in situ hybridization to detect ganglion cell specific gene expression. The coding region of the Ascl1 expression vector and Neurog2 expression vector included a myc epitope. Immunohistochemistry for myc was used to detect transfected cells. Alternatively, in situ hybridization with antisense probe to Ascl1 or Neurog2 was used to detect transfected cells in some tissue sections of electroporated animals. Since transfected cells expressed the ectopically introduced gene at higher level than the endogenous level of expression, in situ

hybridization with low titer of the antisense probe to Ascl1 or Neurog2 specifically detected the transfected cells with no or little detection of endogenous gene expression.

We found that Ascl1 misexpressed cells exhibited characteristics of ganglion cells. The majority of cells with ectopic Ascl1 expression had migrated to the innermost layer of the optic cup (Fig. 6*B*, *Ca*), where the ganglion cells normally would be located. The majority of the Ascl1 misexpressed cells were positive ganglion cell markers including RA4, Isl1, and nTub (Fig. 6*B*, *Ca-Cd*, *D*). In some cases, the proneural gene expression vector was co-electroporated with an expression vector for membrane targeted GFP, which allowed us to examine the overall morphology of the transfected cells. Twenty four hours after electroporation, the electroporated retinas were analyzed histologically. Particularly in flat-mounted retinas, many of the GFP-positive, transfected cells had a long process that resembled an axon (Fig. 6*E*). These data suggest that Ascl1 expression is sufficient to ganglion cell genesis. Neurog2 misexpression induced a ganglion cell like phenotype similar to that seen following Ascl1 misexpression. Neurog2 misexpressed cells also were located in the innermost layer of the retina (Fig. 7*B*, *Ca*). In some cases, an excessive number of transfected cells accumulated on the inner side of the retina and formed a bump into the vitreous chamber (Fig. 7*B*). Most transfected cells expressed the ganglion cell markers, RA4, Isl1, and nTub (Fig. 7*B*, *Ca-Cd*, *D*). Likewise, most of the Neurog2 misexpressing cells had a single long axon-like process (Fig. 7*E*). The processes of the Neurog2 misexpressing cells often extended in random directions rather than growing towards the optic fissure. However, the transfected cells located near the normal neurogenic front usually projected their axons correctly joining with the axons of naturally generated ganglion cells (not shown). It is interesting to note that the morphology of cells with Neurog2 misexpression was different than those with Ascl1 misexpression. As a control, GFP expression vector was electroporated in the

preneurogenic zone of the E3.5 retina and analyzed as above. GFP misexpression did not result in expression of ganglion cell markers including RA4 antigen, Isl1, and nTub, and they did not have axon-like processes (not shown). Together, these results suggest that misexpression of early expressed proneural bHLH genes, Ascl1 or Neurog2 is sufficient to initiate ganglion cell development.

III.C.vi. Neurog2 and Ascl1 Initiate Neurogenesis via Different Mechanism

During normal retinal development, a sequential expression of proneural genes follows expression of Ascl1 and Neurog2 prior to ganglion cell genesis as described above. We asked whether Ascl1 and Neurog2 induce the same proneural gene expression cascade. To address this question, we examined expression of other proneural bHLH genes by cells misexpressing Ascl1 or Neurog2. Ascl1 or Neurog2 expression vectors were electroporated into the peripheral region of the retina as before. After twenty four hours, transfected retinas were processed for in situ hybridization to detect expression of other proneural bHLH factors. Surprisingly, Ascl1 misexpression did not result in expression of other proneural genes studied including Neurog2, Neurod1, Neurod4, and Atoh7 (Fig. 8*Ab*, *B*, *C*), although Ascl1 expression did result in neurogenesis based on RA4, nTub, and Isl1 expression and the presence of an axon-like process (Fig. 6*B*, *Ca-Cd*, *D,* 8*Ab*, *B*). It is particularly interesting that Ascl1 appeared to induce ganglion cell genesis without Atoh7 expression, since it is well established that Atoh7 is a key factor for ganglion cell differentiation (Brown et al., 2001; Liu et al., 2001). This result suggests that Ascl1 may direct ganglion cell genesis via a mechanism independent of Atoh7.

Neurog2 misexpression, in contrast to Ascl1, induced expression of the other proneural genes including Neurod1, Neurod4, and Atoh7, genes that are expressed in normal retinal development after Neurog2 expression (Fig. 8*Db*, *E*, *F*). Expression of

Ascl1 that is normally prior to Neurog2, was not induced by Neurog2 misexpression (Fig. 8*Da*). Induction of proneural gene expression and ganglion cell genesis after misexpression of Neurog2 followed the normal temporal sequence (Table 1). The peripheral region of the right eyes of chick embryos was transfected with a Neurog2 expression vector 4, 6, 9, 12, 15, and 18 hours before they reached E4.5. Double fluorescence in situ hybridization for bHLH factors and immunohistochemistry with the RA4 antibody were performed in the retinal sections. Neurog2 mRNA and myc immunoreactivity were first detected four hours after transfection (not shown). Six hours after electroporation, the majority of the transfected cells had already left the mitotic layer, and many had migrated to the innermost layer of the neural retina. Many of those cells had initiated expression of Neurod4. Expression of other bHLH genes and RA4 immunoreactivity were not detected at this time point. With 9 hrs after electroporation, expression of Atoh7 was induced in addition to neurod4. RA4-positive cells, however, were not detected. RA4-positive cells first appeared 12 hrs following Neurog2 misexpression. These results indicate that Neurod4, Atoh7 and RA4 expression are induced after Neurog2 proteins become available. Expression of Ascl1 was never induced in the Neurog2 misexpressing cells. This suggests that misexpression of Neurog2 can induce the normal sequence of bHLH gene expression. These data collectively suggest that Neurog2 promotes ganglion cell genesis via inducing a bHLH gene cascade, whereas Ascl1 promote ganglion cell genesis presumably using a different pathway.

III.D. Discussion

III.D.i. Initiation of Neurogenesis in the Developing Retina

Progenitor cells in the early developing retina, a part of central nervous system, initially divide without generating postmitotic cells. Generation of postmitotic neurons is initiated later during the development. The mechanism underlying initiation of neurogenesis, however, remains poorly understood. Members of the proneural bHLH family of transcription factors have been found to initiate the cascade of events that transform dividing neural progenitor cells into differentiating neurons in multiple regions of a number of species (reviewed in Bertrand et al., 2002). Although development of most retinal ganglion cells, the first cell type born in the vertebrate retina, requires expression of the proneural gene, Atoh7 (Brown et al., 2001; Liu et al., 2001), misexpression of Atoh7 was not sufficient to initiate differentiation (Liu et al., 2001). This suggests that some other proneural gene or genes initiates differentiation upstream of Atoh7. We made use of the central-peripheral gradient of development across the retina to identify the proneural genes expressed just prior to ganglion cell genesis in chick. Surprisingly, we found four proneural genes in addition to Atoh7 expressed prior to the onset of ganglion cell genesis. We also found all five expressed by at least a subset of newly postmitotic retinal ganglion cells, which suggests that one or more of these factors could initiate the events involved in differentiation. We then employed multiprobe in situ hybridization to compare the timing of expression of each factor. We found that the five proneural genes were consistently expressed in a sequence with Ascl1 and Neurog2 expressed first, followed by Neurod1 and Neurod4 expressed at about the same time, and finally by Atoh7 expressed just before terminal mitosis. Since Ascl1 and Neurog2 are expressed first, we asked whether misexpression of either of these can initiate

neurogenesis in the developing retina. Misexpression of Ascl1 or Neurog2 in preneurogenic retina was sufficient to initiate ganglion cell development. These results indicate that Ascl1 and Neurog2 drive ganglion cell differentiation in the developing chick retina.

Previous studies on the function of proneural genes are consistent with our finding that Ascl1 or Neurog2 is sufficient to initiate neurogenesis in the retina. Expression of several proneural genes including Ascl1 and Neurog2 appears to precede the first appearance of postmitotic cells in developing retina (Brown et al., 1998; Matter-Sadzinski et al., 2001). In addition, forced expression of Neurog2 induced trans-differentiation of retinal pigmented epithelial (RPE) cells as retinal neurons (Yan et al., 2001). Misexpression of Neurod1 in RPE cells also resulted in generation of retinal neurons (Yan and Wang, 2000a, 2000b). It will be interesting to test whether proneural genes expressed prior to ganglion cell genesis other than Ascl1 and Neurog2 are also sufficient to initiate neurogenesis in the retina. Ascl1 and Neurog2 have also been linked to initiating neuronal differentiation in other regions of the developing nervous system. Ascl1 or Neurog2 is expressed in ventricular zone of the developing central nervous system with expression of both proneural genes covering most regions of the developing central nervous system (Guillemot and Joyner, 1993; Gradwohl et al. 1996; Sommer et al. 1996; Ma et al. 1997). Genetic deletion of Ascl1 or Neurog2 resulted in severe loss of neuronal lineages in the developing telencephalon (Casarosa et al., 1999; Horton et al., 1999; Nieto et al., 2001). Therefore, it is possible that proneural activity of Ascl1 or Neurog2 is common to most tissues of the central nervous system.

III.D.ii. Stereotypic Sequence of Proneural Gene Expression Prior to Ganglion Cell Genesis

Five proneural genes examined in this study appear to be expressed prior to ganglion cell genesis in a stereotypic temporal sequence. All five proneural genes were expressed by a subset of progenitor cells in the neurogenic zone and in the transition zone. However, the relative central-peripheral position of most peripherally positioned cell expressing each proneural gene was consistent embryo to embryo and at all stages examined. Early embryonic retina exhibits the central-peripheral gradient in maturation. Thus, the consistent positional difference in expression of each proneural gene indicates that expression of these five proneural genes is initiated in a stereotypic temporal sequence. Ascl1 is expressed the earliest, followed by expression of Neurog2 and by Neurog1 and Neurod4. Atoh7 expression is the last among five proneural genes. Data from Neurog2 misexpression study is also consistent with this finding. Misexpression of Neurog2 induced expression of proneural genes in the same sequence as they appear during normal development.

Another study reported the similar positional difference in expression of proneural genes. The peripheral boundary of Ascl1 expression domain was mapped peripheral to the boundary of expression domain for other proneural genes such as Neurog2 and Atoh7 (Matter-Sadzinski et al., 2005). Study of the ciliary marginal zone (CMZ) of the Xenopus retina also identified the similar differences in the position at which expression of each proneural gene first appears. CMZ is the region at the peripheral margin of the mature retina in fish and amphibians and responsible for the growth of retina throughout the lifetime (Straznicky and Gaze, 1971; Johns 1977; Wetts et al., 1989). The central-peripheral position in the CMZ also exhibits a gradation in maturation similar to that seen in the early embryonic retina. In CMZ, expression of Ascl1 was mapped peripherally to that of other proneural genes (Perron et al., 1998). However, spatial difference in expression of the proneural genes other than Ascl1 was not further resolved in both

studies. We made use of misexpression study and multiprobe in situ hybridization to directly compare expression of a number of proneural genes and further identified a sequential expression of proneural genes preceding ganglion cell genesis.

It is surprising that expression of Ascl1, Neurod1, and Neurod4 precedes ganglion cell genesis. Various combinations of these genes have been linked to specification of the horizontal, amacrine, bipolar, rod and cone cell fates, which are specified after the onset of ganglion cell genesis (reviewed in Ohsawa and Kageyama, 2008). A previous study found that progenitor cells later in retinal development can express combinations of proneural genes linked to specification of earlier retinal cell fates (Trimarchi et al., 2008). The significance of cells co-expressing genes for multiple cell fates is unclear. It could mean that progenitor cells initially express the genes that specify all the fates that could be produced and then subsequently repress all but one. Atoh7 has been shown to repress Neurod1 (Le et al., 2006), which would support this possibility.

III.D.iii. Heterogeneity of Progenitor Cells Prior to the Onset of Neurogenesis

Despite the stereotypic temporal sequence of expression of multiple proneural genes, individual progenitor cells do not appear to go through a fixed cascade of proneural gene expression prior to the onset of differentiation. Rather, progenitor cells express heterogeneous combinations of proneural genes. This diversity among progenitor cells was apparent peripheral to the neurogenic front. Previous work showed that several genes are expressed by subsets of retinal progenitor cells (Alexiades and Cepko, 1997; Brown et al., 1998; Perron and Harris, 2000; Dyer and Cepko, 2001; Gouge et al., 2001; Levine and Green, 2004). Comprehensive gene expression profiling performed at the single cell level also showed extensive heterogeneity among retinal progenitors

(Trimarchi et al., 2008). These studies, however, did not specifically identify progenitor cells in response to local differentiated cells, which facilitates cell diversification (e.g. Reh and Tully, 1986; Waid and McLoon, 1998; Belliveau and Cepko, 1999). Since a different progenitor cells are likely to be influenced by different sets of differentiated cells, it is not surprising that they would move towards heterogeneity in the complex environment of differentiated cells. Our finding that progenitor cells are heterogeneous prior to onset of differentiation is surprising. It remains to be determined whether the original population of retinal progenitor cells is composed of multiple intrinsically different cell lineages or whether this heterogeneity arises later, possibly due to some stochastic mechanism.

III.D.iv. Relationship between Proneural bHLH Transcription Factors and Cell Fate

Retinal ganglion cell differentiation may be initiated by two independent pathways. Neurog2 and Ascl1 appear to be capable of initiating ganglion cell genesis independent of one another. They are the first proneural genes expressed prior to the onset of ganglion cells genesis based on their relative central-peripheral positions. Both were also found to be expressed by early postmitotic cells expressing the RA4 antigen, a ganglion cell marker. Misexpression of either factor in the preneurogenic region of the retina resulted in premature neurogenesis. These 'premature' neurons expressed several ganglion cell markers and had axon-like processes. We found that misexpression of Neurog2 induced expression of several proneural genes including Atoh7, which is consistent with previous studies (Matter-Sadzinski et al., 2005; Hernandez et al., 2007). Misexpression of Ascl1 did not appear to induce expression of the other proneural genes including Atoh7. We did not observed co-expression of Neurog2 and Ascl1, which is consistent with a previous study (Marquardt et al., 2001).

However, an array analysis of gene expression by individual mouse retinal progenitor cells did find rare cases of Neurog2 and Ascl1 co-expression, as well as of Ascl1 and Atoh7 co-expression (Trimarchi et al., 2008). Our findings suggest that Neurog2 and Ascl1 may initiate ganglion cell differentiation independently and that only Neurog2 functions via Atoh7 induction.

Other findings are also consistent with the suggestion that ganglion cells are generated by two gene expression cascades, only one of which is dependent of Atoh7. The majority of ganglion cells failed to develop with genetic deletion of Atoh7 (Brown et al., 2001; Liu et al., 2001). A small number of ganglion cells, however, did develop properly (Liu et al., 2001), suggesting the presence of Atoh7-independent mechanism for ganglion cell development. Recent studies identified the homeobox transcription factors Dlx1 and Dlx2 as regulators of ganglion cell specification. In retina, Dlx1/2 expression is restricted to the ganglion cell layer, and Dlx1/2 double knock out animals exhibited a 20-30% loss of ganglion cells (de Melo et al., 2005). Dlx1/2 expression is independent of the Atoh7/Brn3b pathway (Mu et al., 2004). It is worth noting that Ascl1 expression is necessary for expression of Dlx1/2 in many regions of the developing central nervous system (Casarosa et al., 1999; Letinic et al., 2002; Yun et al., 2002; Andrews et al., 2003; Poitras et al., 2007). Whether this same relationship between Ascl1 and Dlx1/2 applies to the developing retina remains to be tested. Taken together, we propose that Neuorg2 and Ascl1 direct ganglion cell development via two distinct gene expression cascades that are independent of each other. Neurog2 may activate the well established pathway involving Atoh7, and Ascl1 may induce the alternative pathway involving Dlx1/2. Genetic deletion of Ascl1 did not have a detectable effect on retinal ganglion cell genesis (Tomita et al., 1996). This could have been because the effect was small and not noticed or because the larger Ngn2/Atoh7 pathway was able to compensate. Retinal ganglion

cells have been classified into a number of subtypes (Sun et al., 2002; Badea and Nathans, 2004; Kong et al., 2005). It will be interesting to determine whether these different gene cascades are linked to development of different retinal ganglion cell subtypes.

Figure III-1. Expression of Proneural Genes Precedes the Generation of Retinal Ganglion Cells Expression of multiple proneural genes was examined relative to the neurogenic front on cross sections of stage 26 chick retinas by fluorescent in situ hybridization. Immunohistochemistry with the RA4 antibody (green) on the same sections was used to labele retinal ganglion cells, the first cell type generated in the retina. The presence of RA4-positive cells defines the neurogenic zone, the region with ongoing neurogenesis. The green arrowhead in each micrograph indicates the position of the peripheral-most RA4-positive cell, the neurogenic front. Red arrowheads indicate the peripheral boundary of expression of relevant proneural gene. DAPI staining was used to label all nuclei (cyan). In micrographs ***A*** and ***B***, the temporal side of the retina is towards the bottom and the nasal side is towards the top. ***A***, Sox2 expression (red) identifies neural progenitor cells. At stage 26, most cells in the retina were Sox2-positive. Cells in the peripheral margin of the optic cup, the ciliary epithelium, were negative for Sox2. The open arrowheads indicate the boundary between the Sox2-positive neural retina and the ciliary epithelium. RA4-positive ganglion cells (green) were negative for Sox2. ***B***, Delta1 (cyan) was expressed by preneurogenic progenitor cells and by newly formed postmitotic cells in the neurogenic zone. Just peripheral to the neurogenic front, Delta1 expression was gradually down-regulated. Ascl1 (red), a proneural gene, was expressed by a subset of progenitor cells in the neurogenic zone. Ascl1 expression first came up just peripheral to the neurogenic front in the region where Delta1 expression was downregulated. ***C***-***G***, High magnification micrographs show expression of a number of proneural genes in a region corresponding to the boxed area in micrograph ***B***. Proneural genes examined include Ascl1 (***C***), Atoh7 (***D***), Neurod1 (***E***), Neurod4 (***F***), and Neurog2 (***G***). The onset of expression of all five proneural genes was peripheral to the neurogenic front. Scale bars: ***A***-***B***, 100 μm; ***C***-***G***, 50 μm.

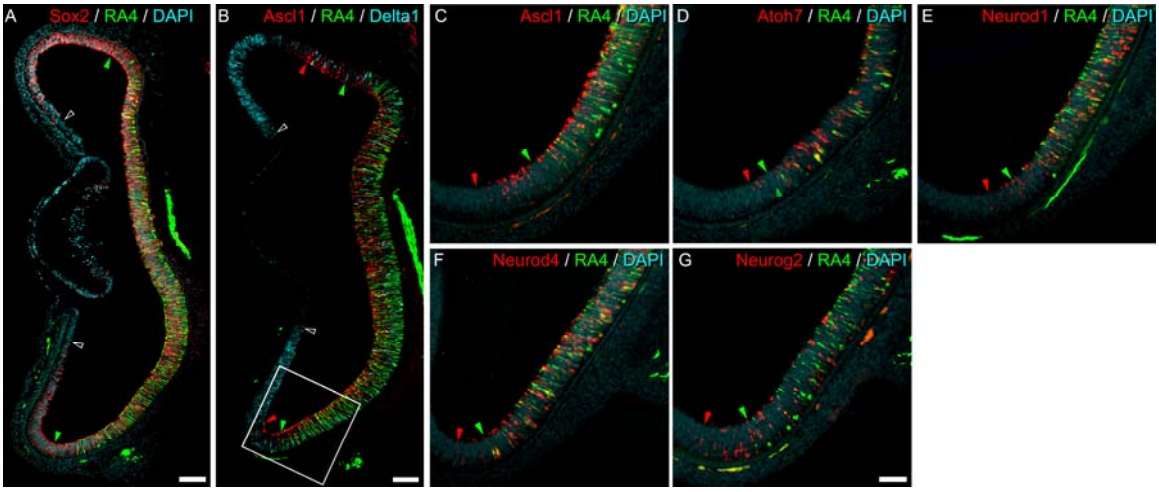

Figure III-1. Expression of Proneural Genes Precedes the Generation of Retinal Ganglion Cells

Figure III-2. Newly Generated Retinal Ganglion Cells Express Multiple Proneural Genes Micrographs show a region of the neurogenic zone of stage 26 chick retina with the mitotic layer towards the bottom and the inner retina towards the top. Fluorescent in situ hybridization shows expression of several proneural genes (red). The proneural genes examined include Ascl1 (***A***), Atoh7 (***B***), Neurod1 (***C***), Neurod4 (***D***), and Neurog2 (***E***). RA4 immunoreactivity (green) identifies the retinal ganglion cells. DAPI staining labels all nuclei (cyan). All five proneural genes were expressed at least by a subset of newly postmitotic, RA4-positive ganglion cells in and near the mitotic layer. Asterisks indicate the nuclei of some RA4-positive cells that also express proneural genes. Scale bar: 10 μm.

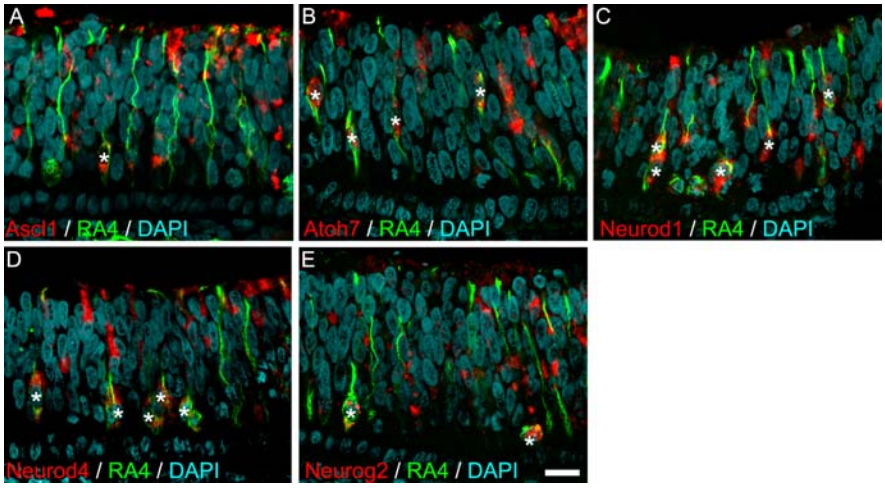

Figure III-2. Newly Generated Retinal Ganglion Cells Express Multiple Proneural Genes

Figure III-3. Stereotypic Temporal Sequence of Proneural Gene Expression Prior to Ganglion Cell Genesis Expression of two proneural genes (red and green) were compared in a section by multi-probe fluorescence in situ hybridization. Each micrograph shows a region of stage 26 chick retina surrounding the neurogenic front. Immunoreactivity for RA4 antigen or neuronal tubulin (nTub) identified ganglion cells (cyan) and the position of the neurogenic front (cyan arrowheads). Note that the most peripheral position of nTub expression is more centrally positioned than that for RA4, reflecting the slightly later onset of nTub expression compared to RA4 expression. The most peripherally positioned cells expressing each proneural gene are marked by the red and green arrowheads. ***A***, The most peripherally positioned cell expressing Ascl1 (green) was consistently located more peripheral to that for Nerurog2 (red). ***B***, The most peripherally positioned cell expressing Neurog2 (red) was consistently positioned more peripheral to that for Neurod1 (green). ***C***, Neurod1 and Neurod4 were expressed at about the same position. ***D***, The most peripherally positioned cell expressing Neurod1 (red) was consistently positioned more peripheral to that for Atoh7 (green). Expression of all five proneural genes was always found peripheral to the neurogenic front. Scale bar: 50 μm. All micrographs are at the same magnification.

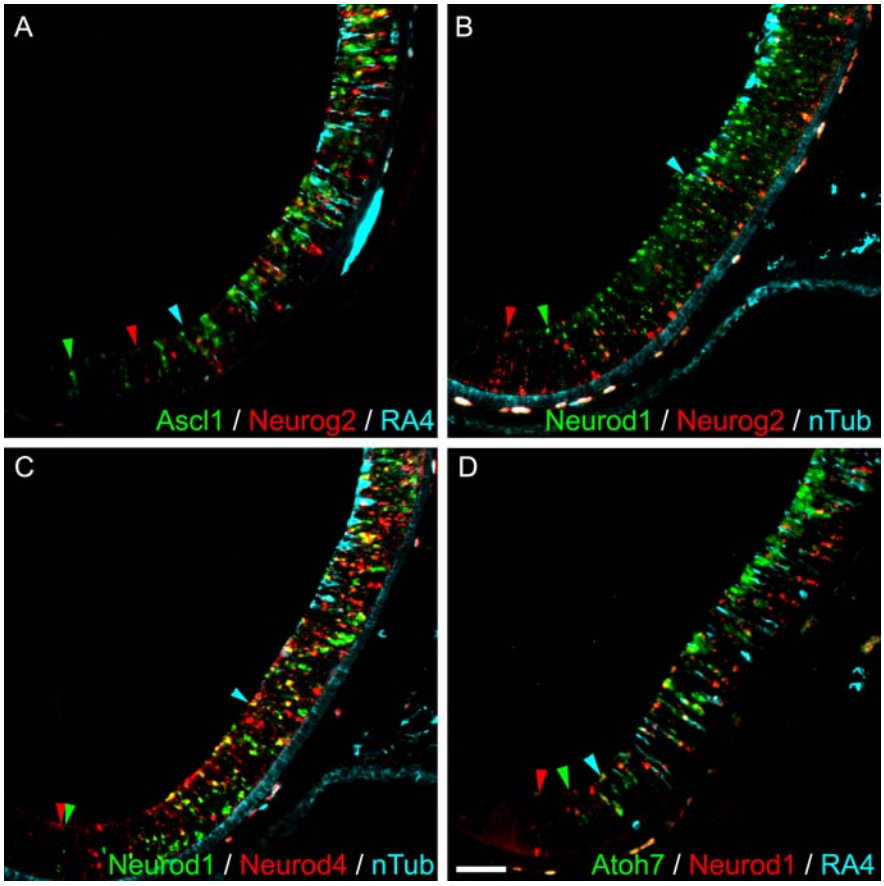

Figure III-3. Stereotypic Temporal Sequence of Proneural Gene Expression Prior to Ganglion Cell Genesis

Figure III-4. Progenitor Cell Heterogeneity Prior to the Onset of Neurogenesis Micrographs show the retina just peripheral to the neurogenic front with the mitotic layer at the bottom and the inner layer at the top. Double fluorescence in situ hybridization on the stage 26 chick retina shows the expression of two proneural genes (red and green). Nuclei of the cells expressing one gene of each pair are marked with red or green asterisks, and nuclei of the cells expressing both genes are marked with yellow asterisks. DAPI staining labels all nuclei (cyan). Micrographs in a row show the identical field of view with DAPI staining omitted in the micrographs on the right. ***Aa-Bb***, Expression of Neurod1 or neurod4 was compared to expression of Atoh7, the last proneural gene expressed before the onset of neurogenesis. Atoh7-positive cells (red) in the transition zone were found both with (yellow) and without (red) Neurod1 or Neurod4 expression. Some Neurod1- or Neurod4-positive cells were negative for Atoh7 expression (green). ***Ca-Cb***, Neurod1 and Neurod4 were expressed by overlapping but distinct populations of progenitor cells. Some cells in the transition zone expressed either Neurod1 (green) or Neurod4 (red), and some cells expressed both genes (yellow). ***Da-Eb***, Expression of Ascl1 or Neurog2 was compared to expression of Atoh7. None of Atoh7-positive cells (red) in the transition zone co-expressed Ascl1 (green). Atoh7-positive cells (red) in the transition zone were found both with (yellow) and without (red) Neurog2 expression. Some Neurog2-positive cells were negative for Atoh7 expression (green). ***Fa-Fb***, Ascl1 and Neurog2 were expressed by distinct populations of progenitor cells. Scale bar: 10 μm. All micrographs are at the same magnification.

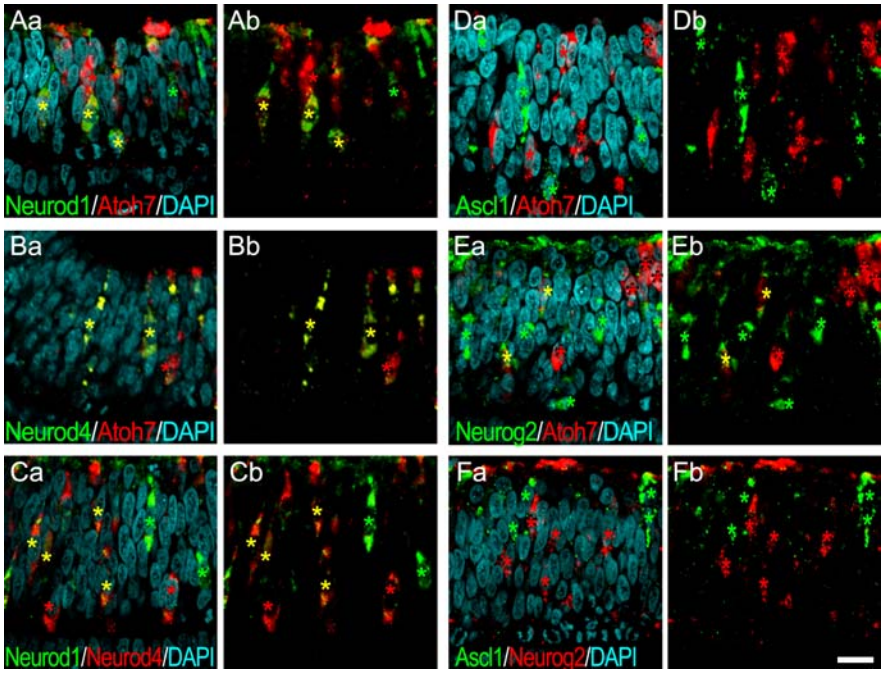

Figure III-4. Progenitor Cell Heterogeneity Prior to the Onset of Neurogenesis

Figure III-5. Proneural Genes Expressed by RA4 or Atoh7 Positive Cells *A*, Dissociated cells from the central region of stage 26 chick retina were processed for in situ hybridization to detect expression of each proneural gene and immunohistochemistry with RA4 antibody to identify newly differentiating ganglion cells. The percentage of RA4-positive cells that did and did not express each proneural gene was plotted in gray and white, respectively. ***B***, Dissociated cells from the central region of stage 26 chick retina were processed for double fluorescence in situ hybridization to identify proneural genes expressed by Atoh7-positive cells. The percentage of Atoh7-positive cells that did and did not express other proneural genes was plotted in gray and white, respectively.

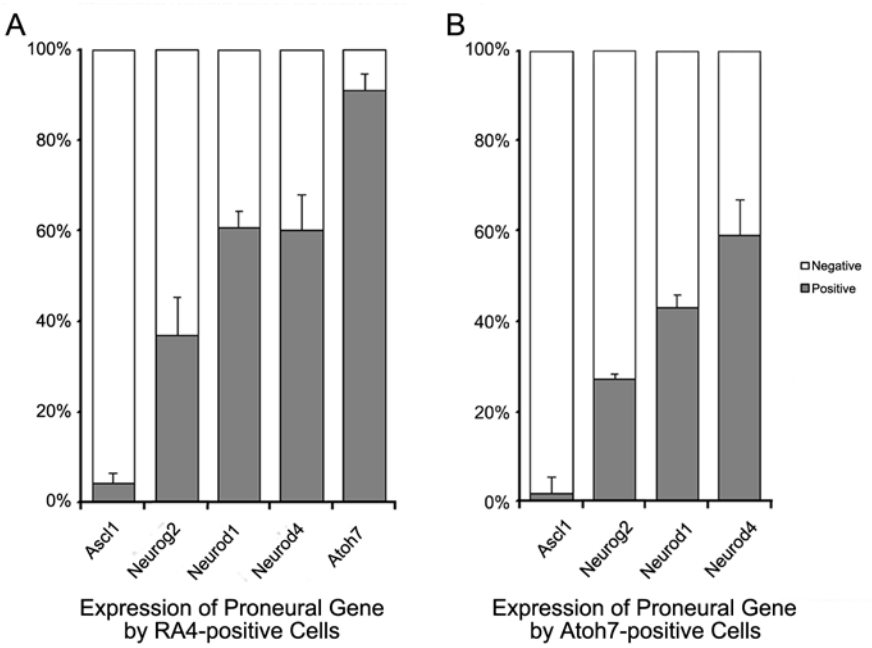

Figure III-5. Proneural Genes Expressed by RA4 or Atoh7 Positive Cells

Figure III-6. Premature Neurogenesis Induced by Ascl1 Misexpression Cells in the peripheral retina of E3.5 embryos were electroporated in ovo with an Ascl1 expression vector. In some cases, an expression vector for membrane targeted GFP was co-electroporated. Twenty four hours later, transfected cells expressed neuronal markers consistent with ganglion cell differentiation. ***A***, Transfected cells (green) in the peripheral retina expressed the RA4 antigen (red), a marker for differentiating ganglion cells. Most Ascl1 misexpressing cells had migrated to the inner retina. ***B***, No RA4-positive cells were present in the periphery of the untreated, contralateral retina from the same embryo. The orientation of the micrograph in ***B*** was flipped so as to match the orientation of the retina in ***A***. The red arrowheads in ***A*** and ***B*** indicate the most peripherally positioned RA4-positive cells. ***Ca***-***Cd***, Micrographs ***Ca***-***Cd*** show high magnification images of a region of Ascl1 misexpression. A cross section of an Ascl1 misexpressing retina was processed for double fluorescent in situ hybridization to detect cells misexpressing Ascl1 (green) and Isl1 expression (red) and for immunohistochemistry with anti-nTub antibody (blue). Expression of Isl1 and nTub labels ganglion cells. Each macrograph shows different combinations of channels of the same cross section and the same filed of view. Merged image is shown without DAPI in micrograph ***Cd***. Asterisks indicate the nuclei of the Ascl1 misexpressing cells co-expressing Isl1 and nTub. ***D***, A plot of a retinal flat mount shows the distribution of cells that co-expressed GFP and the RA4 antigen (green) following co-transfection with Ascl1 and membrane targeted GFP expression vectors. The area of the retina with natural neurogenesis is indicated in red (i.e. RA4-positive and GFP-negative). ***E***, This micrograph shows GFP-labeled cells (green) in the boxed region of ***D***. Many transfected cells had a single axon-like process. Scale bars: ***A***-***B***, 100 μm; ***Ca***-***Cd***, 25 μm; ***D***, 250 μm; ***E***, 50 μm. Micrographs ***A*** and ***B*** are at the same magnification.

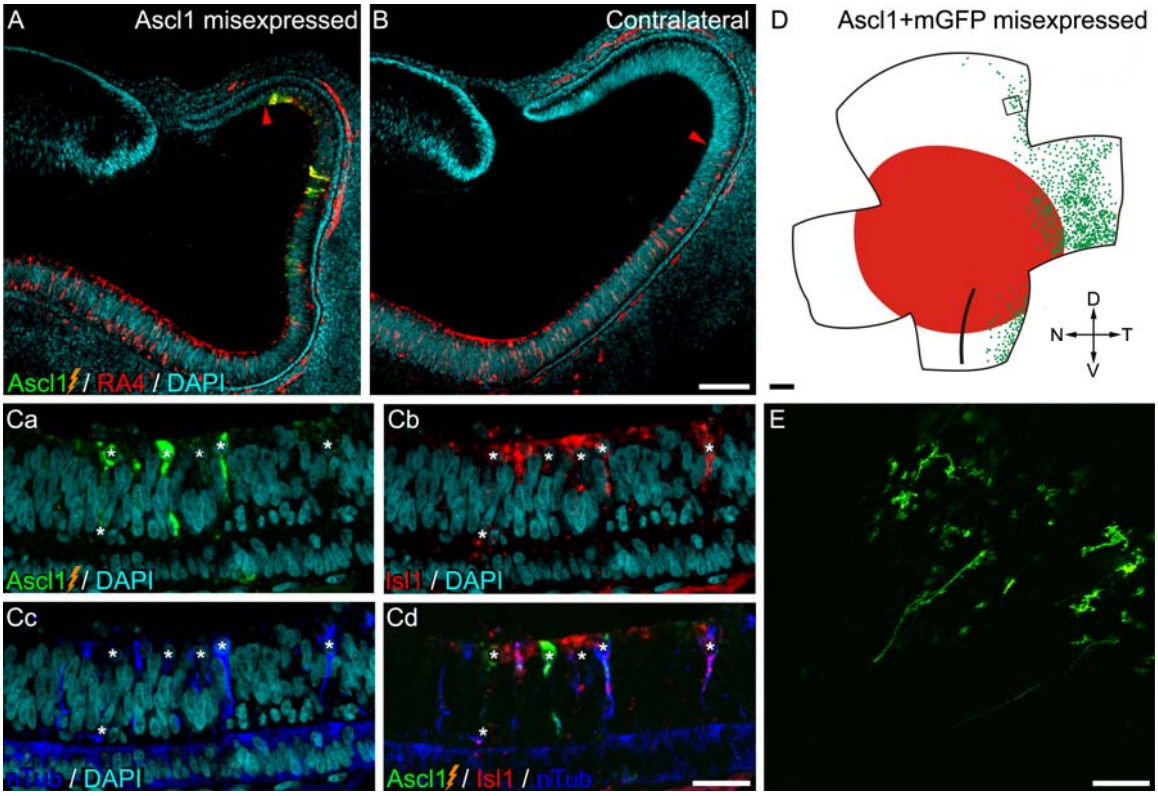

Figure III-6. Premature Neurogenesis Induced by Ascl1 Misexpression

Figure III-7. **Premature Neurogenesis Induced by Neurog2 Misexpression** Cells in the peripheral retina of E3.5 embryos were electroporated in ovo with a Neurog2 expression vector. In some cases, an expression vector for membrane targeted GFP was co-electroporated. Twenty four hours later, transfected cells expressed neuronal markers consistent with ganglion cell differentiation. ***A***, Transfected cells (green) in the peripheral retina expressed the RA4 antigen (red), a marker for differentiating ganglion cells. Most Neurog2 misexpressing cells had migrated to the inner retina. ***B***, No RA4-positive cells were present in the periphery of the untreated, contralateral retina from the same embryo. The orientation of the micrograph in ***B*** was flipped so as to match the orientation of the retina in ***A***. The red arrowheads in ***A*** and ***B*** indicate the most peripherally positioned RA4-positive cells. ***Ca***-***Cd***, Micrographs ***Ca***-***Cd*** show high magnification images of a region of Neurog2 misexpression. A cross section of a Neurog2 misexpressing retina was processed for double fluorescent in situ hybridization to detect cells misexpressing Neurog2 (green) and Isl1 expression (red) and for immunohistochemistry with anti-nTub antibody (blue). Expression of Isl1 and nTub labels ganglion cells. Each macrograph shows different combinations of channels of the same cross section and the same filed of view. Merged image is shown without DAPI in micrograph ***Cd***. Asterisks indicate the nuclei of the Neurog2 misexpressing cells co-expressing Isl1 and nTub. ***D***, A plot of a retinal flat mount shows the distribution of cells that co-expressed GFP and the RA4 antigen (green) following co-transfection with Neurog2 and membrane targeted GFP expression vectors. The area of the retina with natural neurogenesis is indicated in red (i.e. RA4-positive and GFP-negative). ***E***, This micrograph shows GFP-labeled cells (green) in the boxed region of ***D***. Many transfected cells had a single axon-like process. Scale bars: ***A***-***B***, 100 μm; ***Ca***-***Cd***, 25 μm; ***D***, 250 μm; ***E***, 50 μm. Micrographs ***A*** and ***B*** are at the same magnification.

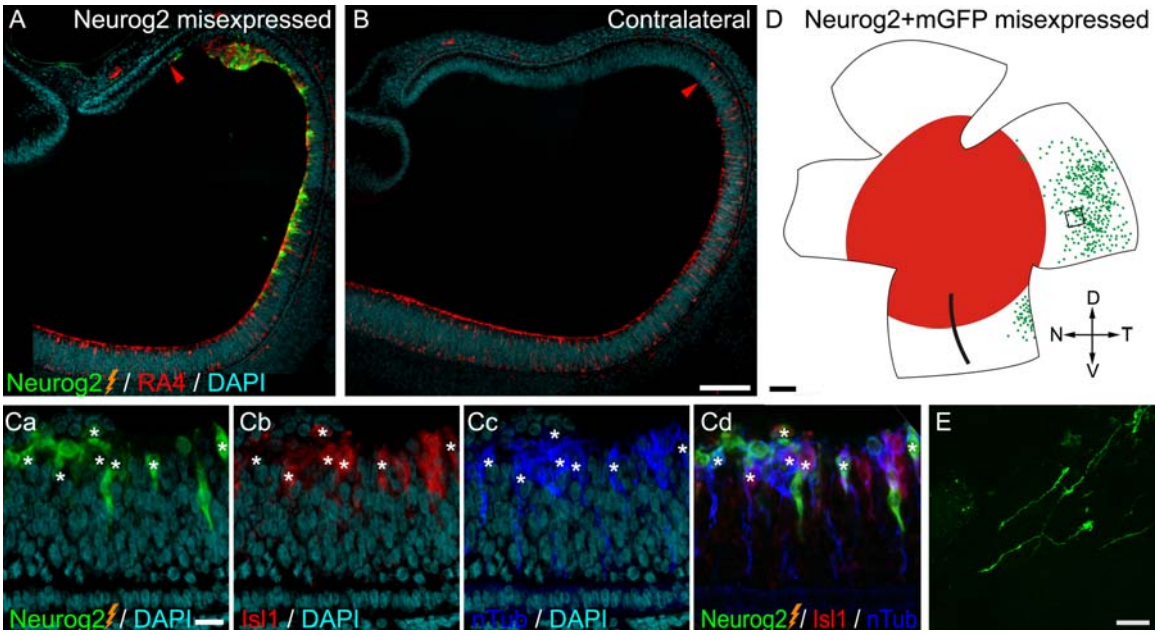

Figure III-7. Premature Neurogenesis Induced by Neurog2 Misexpression

Figure III-8. Differential Induction of Proneural Gene Expression by Ascl1 or Neurog2 Misexpression Cells in the peripheral retina of E3.5 embryos were electroporated in ovo with an Ascl1 or HA-tagged Neurog2 expression vector. Twenty-four hours later, the proneural genes expressed by the transfected cell was determined histologically using in situ hybridization. ***Aa***-***C***, Micrographs show the same or neighboring sections of a retina with Ascl1 misexpressed. Ascl1 misexpressing cells (green) expressed RA4, ganglion cell marker (red). None of the Ascl1 misexpressing cells expressed other proneural genes including Neurod4 (cyan in ***Ab***), Neurog2 (cyan in ***B***), Neurod1 and Atoh7 (cyan and red, respectively, in ***C***). ***Da***-***Hb***, Cells misexpressing Neurog2 were detected by either HA immunoreactivity or Neurog2 in situ hybridization. ***D***, Cells misexpressing Neurog2 (green) expressed the RA4 antigen, a ganglion cell marker. ***E***, Cells misexpressing Neurog2 (green) did not express Ascl1 (red), a proneural gene normally expressed earlier than Neurog2. ***F***-***H***, Most cells misexpressing Neurog2 also expressed other proneural genes including Atoh7, Neurod1, and Neurod4 (red). Scale bars: ***C***, 100 μm; ***G***, 50 μm. Micrographs in a row are at the same magnification.

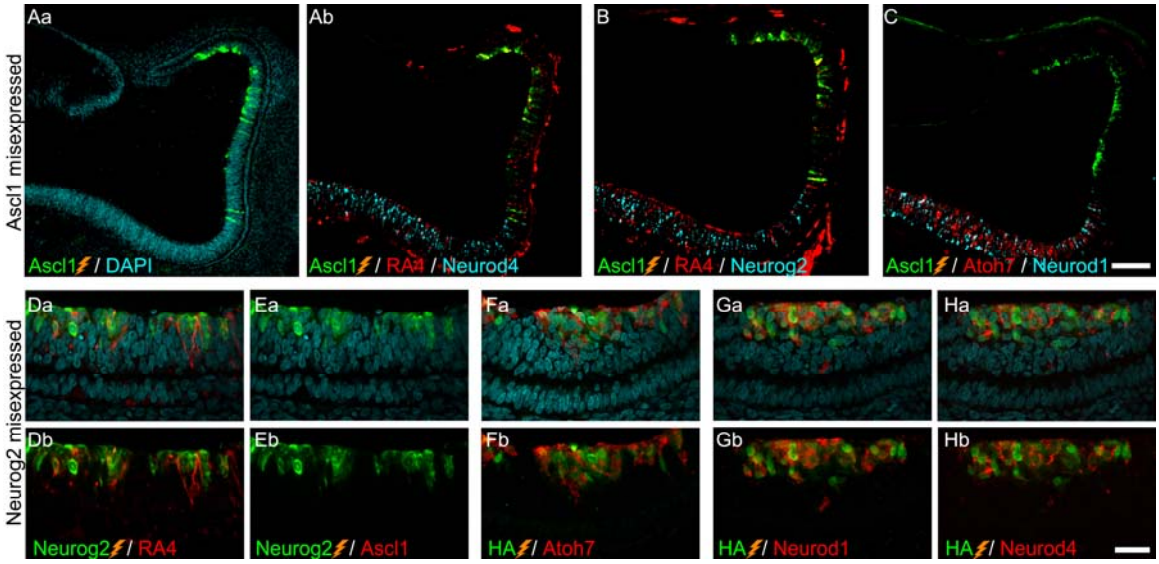

Figure III-8. Differential Induction of Proneural Gene Expression by Ascl1 or Neurog2 Misexpression

Table III-1. Sequential Induction of Proneural Gene Expression Following Neurog2 Misexpression The preneurogenic zone of E3.5 to E4.5 retinas was electroporated in ovo with a Myc-tagged Neurog2 expression vector. The electroporated embryos were then incubated for 6, 9, 12, 18, 24 hours until they reached E4.5 of age. Then the retinas were analyzed histologically to identify the proneural genes expressed by the transfected cells. '+' or '-' indicates co-expression or the lack of expression respectively.

Post Transfection Survival Time	Gene Expression by Cells Misexpressing Neurog2				
	Ascl1	Neurod1	Neurod4	Atoh7	RA4
6 hours	-	+	+	-	-
9 hours	-	+	+	+	-
12 hours	-	+	+	+	+
18 hours	-	+	+	+	+
24 hours	-	+	+	+	+

Table III-1. Sequential Induction of Proneural Gene Expression Following Neurog2 Misexpression

CHAPTER IV.

CONCLUSIONS AND FUTURE DIRECTIONS

IV.A. Conclusions

The initial maintenance of preneurogenic progenitor cells and the precise regulation of initiation of neurogenesis are essential for the proper development of the vertebrate central nervous system. The goal of the work described in this thesis was to understand the mechanisms underlying these processes. Using the developing chick retina as a model, the following questions were addressed: 1) what are the molecular differences between preneurogenic and neurogenic progenitor cells, 2) what regulates progenitor cell maturation from preneurogenic to neurogenic, and 3) what initiates neuronal differentiation in the neurogenic progenitor cells? The main findings of this thesis are summarized below.

IV.A.i Molecular Distinction between Preneurogenic and Neurogenic Progenitor Cells

Studies in chapter II showed that preneurogenic and neurogenic progenitor cells are molecularly distinct in the vertebrate retina. All cells in the preneurogenic zone of the retina express Delta1, a ligand for the Notch receptor. Progenitor cells in the neurogenic zone do not express Delta1. Progenitor cells in the neurogenic zone express E2A, which encodes a binding partner for proneural bHLH transcription factors and is required for neuronal differentiation. During the preneurogenic to neurogenic transition, progenitor cells down-regulate Delta1 expression and up-regulate E2A expression. All progenitor cells express Sox2, a neural progenitor cell marker, regardless of the maturation state. Therefore, Delta1/Sox2 expression identifies preneurogenic progenitor cells whereas E2A/Sox2 expression identifies the neurogenic progenitor cells in the developing vertebrate retina. These data suggest that the progenitor cells that exhibit preneurogenic

and neurogenic modes of division are fundamentally different. This is the first time that molecular markers specific to preneurogenic and neurogenic progenitor cell populations have been identified. It is important to note that all cells in a given region of the retina down-regulate Delta1 expression and up-regulate E2A expression prior to appearance of the first post-mitotic cell in a region. Furthermore, dividing cells do not appear to lose E2A expression and reacquire Delta1 expression once differentiation has commenced. Taken together, these findings suggest that the transition of progenitor cells from preneurogenic to neurogenic is a unidirectional maturation step made in unison by neighboring progenitor cells. It remains to be determined whether progenitor cells in other tissues of the developing central nervous system display the same molecular distinction between preneurogenic and neurogenic stages.

IV.A.ii. Control of Progenitor Cell Maturation

The study described in chapter II suggests that Delta-Notch signaling maintains preneurogenic progenitor cells, whereas Shh, released from newly formed ganglion cells, promotes progenitor cell maturation from preneurogenic to neurogenic. Interrupting Notch signaling in the preneurogenic population resulted in loss of Delta1 expression, up-regulation of E2A expression and the premature onset of neurogenesis. This suggests that blocking Notch signaling converted progenitor cells, which would normally be preneurogenic, into neurogenic progenitor cells. Shh normally is expressed by a subset of newly differentiating ganglion cells. Misexpression of Shh in the preneurogenic progenitor cells initiated the preneurogenic to neurogenic transition including the down-regulation of Delta1 expression. Collectively, these data suggest that the preneurogenic mode of division is initially maintained by active Notch signaling and that progenitor cell maturation is initiated by a cell extrinsic mechanism mediated by Shh. Whether Shh

promote progenitor cell maturation by interfering with Notch signaling still remains to be investigated.

IV.A.iii. Initiation of Neurogenesis by Proneural bHLH Transcription Factors in the Developing Vertebrate Retina

The study presented in chapter III investigated the role of bHLH transcription factors in initiation of neurogenesis. Characterization of proneural gene expression revealed that multiple proneural genes are expressed just prior to the first appearance of postmitotic cells. Ascl1, Atoh7, Neurod1, Neurod4, and Neurog2 were expressed in the transition zone, in a region just peripheral to the neurogenic front. This region contains the progenitor cells that are preparing for neuronal differentiation. Interestingly, multiple proneural genes are expressed in a stereotypic temporal sequence: Ascl1, Neurog2, Neurod1 & Neurod4, and Atoh7. Individual progenitor cells in the transition zone appeared to express heterogeneous combinations of proneural genes. The importance of heterogeneity among progenitor cells of the transition zone remains to be determined. These data are consistent with the prediction that progenitor cells undergo successive up-regulation of multiple proneural bHLH transcription factors preceding neurogenesis.

We then asked whether any of the proneural genes expressed prior to neurogenesis are sufficient to initiate neurogenesis. Misexpression of Ascl1 or Neurog2 was sufficient to induce ganglion cell genesis. Cells transfected with Ascl1 or Neurog2 expression vectors expressed multiple markers for ganglion cells and had long processes similar to axons of ganglion cells. Misexpression of Neurog2 initiated the stereotypic sequence of proneural gene expression that normally precedes ganglion cell genesis. Whether the sequential up-regulation of proneural genes in individual progenitor cells is required for neuronal differentiation was not tested in this study.

Further investigation will be necessary to address this issue. In contrast to Neurog2, Ascl1 appears to initiate ganglion cell genesis by using a different molecular mechanism than does Neurog2. Although Ascl1 expression was sufficient to initiate the ganglion cell genesis, it did not induce expression of other proneural genes. Taken together, the results of misexpression studies showed that Ascl1 or Neurog2 is sufficient to initiate ganglion cell genesis. Data also suggest that Ascl1 and Neurog2 initiate ganglion cell genesis via different mechanisms.

IV.B. Future Directions

IV.B.i. Differential Function of Notch Signaling in Two Progenitor Cell Stages

As shown in chapter II, Delta-Notch signaling maintains the preneurogenic mode of division. In the preneurogenic zone of the retina, all cells express the ligand, Delta1. This suggests that Notch activation is reciprocal between adjacent progenitor cells. Reducing Notch activity in this region resulted in conversion of preneurogenic progenitor cells to neurogenic and premature appearance of differentiating cells. In contrast, in the neurogenic zone of the developing retina, Delta1 is expressed only by isolated newly postmitotic cells, so Notch signaling is uni-directional. Previous studies from our laboratory showed that knockdown of Notch expression increased production of ganglion cells at the expense of cone cells during early neurogenesis in chick retina (unpublished). Reducing Notch activity did not affect cell division in the neurogenic zone. Some previous studies also were unable to link Notch activity to retinal cell division (Austin et al., 1995; Bao and Cepko, 1997; Silva et al., 2003). The main function of Notch signaling in neurogenic progenitor cells appears to be to influence cell fate decisions. Collectively, it appears that the two different modes of Notch signaling have distinct functions in preneurogenic and neurogenic zones of the retina.

A testable hypothesis is that the different roles of Notch in preneurogenic and neurogenic retina are due in part to expression of different components on the Notch signaling system in the two areas. Several components of Notch signaling appear to be differentially expressed in preneurogenic and neurogenic zone. Hes1 and Hes5 are expressed upon activation of Notch signaling and known to mediate the function of Notch activation (Nishimura et al., 1998; Kageyama et al., 2007). Interestingly, preliminary data from our laboratory showed that Hes5 is expressed by neurogenic

progenitor cells but not by preneurogenic progenitor cells. Data from other groups showed that Hes1 is expressed by most cells in the peripheral region of the early developing retina whereas only a small number of isolated cells express Hes1 (Brown et al., 1998; Matter-Sadzinski et al., 2005). It will be interesting to test whether Hes1 and Hes5 mediate different functions of Notch signaling in preneurogenic and neurogenic zones.

IV.B.ii. Ascl1 Initiated Gene Expression Cascade and Retinal Ganglion Cell Subtype Specification

Misexpression studies in chapter III suggest the possibility that the ganglion cells are generated through at least two independent mechanisms, one initiated by Ascl1 and another initiated by Neurog2. Atoh7 is believed to be a key factor for ganglion cell development (Brown et al., 2001; Liu et al., 2001). Consistent with this, Atoh7 expression was induced by Neurog2 misexpression prior to the generation of ganglion cells. However, it is surprising that Ascl1 expression promoted ganglion cell genesis without inducing expression of Atoh7. This finding suggests that the gene expression cascade for ganglion cell genesis initiated by Ascl1 is independent of Atoh7. The molecular mechanism by which Ascl1 initiates ganglion cell genesis remains to be determined.

Although it is often overlooked, genetic deletion of Atoh7 did not result in a complete failure of ganglion cells to develop (Brown et al., 2001; Liu et al., 2001; Lin et al., 2004). This suggests that a factor other than Atoh7 also accounts for ganglion cell development. Recent studies have implicated the homeobox transcription factor Distal-less 1 (Dlx1)/Dlx2 in ganglion cell development. Dlx1 and Dlx2 expression is restricted to the ganglion cell layer, and the Dlx1/Dlx2 double knock out mice exhibited a partial loss

of ganglion cells (de Melo et al., 2005). Expression of Brn3b, a POU domain transcription factor, is downstream of Atoh7 expression, and a null mutation of Brn3b results in a significant loss of ganglion cells (Erkman et al., 1996; Gan et al., 1996; Lin 2004). However, Dlx1/Dlx2 was still expressed in the Brn3b mutant retina (Mu et al., 2004). It is worth noting that Ascl1 expression is necessary for expression of Dlx1 and Dlx2 in many regions of central nervous system (Cassarosa et al., 1999; Letinic et al., 2002; Yun et al., 2002; Andrews 2003). Based on these results, it can be proposed that Ascl1 initiates ganglion cell genesis via a gene expression cascade involving Dlx1/Dlx2. The data in chapter III showing that Ascl1 initiated ganglion cell genesis without inducing expression of Atoh7 is consistent with this possibility.

Studies suggest that retinal ganglion cells can be further grouped into a number of distinct subtypes (Sun et al., 2002; Badea and Nathans, 2004; Kong et al., 2005). It is reasonable to predict that a certain subtype(s) of ganglion cells are specified by a mechanism involving Ascl1 and Dlx1/Dlx2. Identifying the subtypes of ganglion cells in the lineage of Ascl1 expressing cells in the mature retina could test this hypothesis. Mouse genetics could be employed to follow the lineage of Ascl1 expressing cells. In animals that carry a gene encoding Cre recombinase under control of Ascl1 promoter and a reporter gene, LoxP-Stop-LoxP-GFP, the cells, which once expressed Ascl1, will continuously express GFP. This will allow identification of the Ascl1 lineage cells in mature animals even after cells have terminated Ascl1 expression. Immunohistochemistry or in situ hybridization to show expression of markers for ganglion cell subtypes could be used to identify the ganglion cell subtypes in the Ascl1 lineage. Using the same technique, the short-term tracing of the Ascl1 lineage also would be possible. This could show whether Ascl1 leads to expression of Dlx1/Dlx2 and then only certain subtype of ganglion cells.

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