NEUROGENESIS AND GLIOGENESIS FROM ASCL1 (MASH1) EXPRESSING PROGENITORS IN THE CNS

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To my mom

for her unimaginable love, support and devotion throughout my life.

NEUROGENESIS AND GLIOGENSIS FROM ASCL1 (MASH1) EXPRESSING PROGENITORS IN THE CNS

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For the functional architecture of the central nervous system, a small population of neural stem cells generates the correct numbers and types of neurons, oligodendrocytes and astrocytes in a precisely coordinated manner. Basic helix-loop-helix (bHLH) transcription factors play central roles in determining distinct neural cell fates and thus contribute to mechanisms controlling neural cell type diversity during the embryogenesis. Fundamental to understanding nervous system formation is to uncover links between early cell type specification mechanisms, the developmental dynamics of each lineage, and the contributions of specific molecules to these processes to form the mature nervous system structures.

Ascl1 (previously Mash1) is a bHLH transcription factor essential for neuronal differentiation and neural sub-type specification. Ascl1 is present in proliferating progenitor cells but these cells are actively differentiating as evidenced by their rapid migration out of germinal zones. Although it has been studied for its role in several neural lineages, the full complement of lineages arising from Ascl1 progenitor cells and the molecular mechanism of Ascl1's functions are not completely understood. Using an inducible Cre-flox genetic fate-mapping strategy, Ascl1 lineages were determined in both the embryonic and adult central nervous system. In chapter two, the fate of $Ascl1^{+}$ progenitor cells throughout the brain was described. Depending on the temporal and spatial context during embryogenesis, $Ascl1^{+}$ cells contribute to distinct neuronal and glial cells in each major brain division. In chapter three, by labeling $Ascl1^{+}$ progenitor cells at distinct phases of their development, I delineated the temporal lineage relationship of distinct subtypes of neurons and glia in the developing spinal cord. Two spatially and temporally distinct $Ascl1^{+}$ progenitor populations contribute differentially to inhibitory dIL^{A} and excitatory dIL^{B} neurons in the dorsal spinal cord. At later stages of embryogenesis, $Ascl1^{+}$ progenitors are restricted to glial lineages giving rise to both astrocytes and oligodendrocytes. Analysis of conditional mutants of *Ascl1* demonstrated that Ascl1 is required for only one division of each lineage. Loss of Ascl1 results in a reduction of inhibitory dIL^{A} neurons and oligodendrocytes, but not excitatory dIL^{B} neurons and astrocytes. In chapter four, the physiological functions of Nicastrin in gliogenesis were investigated. Nicastrin is a requisite subunit of the γ-secretase complex essential for activating Notch signaling pathway. Conditional mutant of Nicastrin leads to the increased level of oligodendrocytes lineage markers in the neural tube, the opposite

phenotype of that for Ascl1. Thus, I propose that Notch signaling in constraining levels of Ascl1 is required in oligodendrogenesis. In chapter five, I revealed that Ascl1 is a common molecular marker of early progenitors of both neurons and oligodendrocytes in the adult brain, and these Ascl1 defined progenitors mature with distinct dynamics in different brain regions. In this thesis, I define Ascl1 as a neural differentiation factor crucial for neural cell type diversification, playing important roles in cell differentiation and subtype specification at several different nodes of cell fate decisions throughout neurodevelopment.

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PRIOR PUBLICATIONS

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LIST OF ABBREVIATIONS

4v	fourth ventricle
AEP	anterior entopeduncular area
AH	Ammon's horn of hippocampus
Amyg	amygdala
AOB	accessory olfactory bulb
APC	adenomatous polyposis coli
Ascl1	achaete-scute homolog 1
Atoh1	atonal homolog 1
BAC	bacterial artificial chromosome
bHLH	basic helix-loop-helix
BLBP	brain-lipid-binding protein
BMP	bone morphogenetic protein
cc	corpus callosum
CGE	caudal ganglionic eminence
cKO	conditional knockout
CN	cochlear nucleus
CNS	central nervous system
Ctx	cortex
CTZ	cortical transitory zone
DCN	deep cerebellar nuclei
Dcx	doublecortin
DG	dentate gyrus
dI	dorsal interneuron
dIL	dorsal interneuron late
dIL^A	dorsal interneuron late A
dIL^B	dorsal interneuron late B
Dll1	deltalike1
Dll3	deltalike3
DNA	deoxyribonucleic acid
dpc	day post coitum
dVZ	dorsal ventricular zone
E	embryonic day
ECN	external cuneate nucleus
Epi	epithalamus
ES	embryonic stem
FACS	fluorescence-activated cell sorting
FGF	fibroblast growth factor
fl	flox
GABA	γ-Aminobutyric acid

GCL	granule cell layer
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
Gi	gigantocellular reticular nucleus
GL	glomerular layer
GLAST	astrocyte-specific glutamate transporter
gm	gray matter
H	harvest
Hb	habenula
HES	hairy/enhancer of split
HI	hippocampus
Hp	hippocampus
Hyp	hypothalamus
Ic	inferior colliculus
ION	inferior olive nucleus
LGE	lateral ganglionic eminence
LPGi	lateral paragigantocellular nucleus
LRL	lower rhombic lip
LRt	lateral reticular nucleus
Maml	mastermind-like
Mash1	mammalian homolog of achaete-scute complex 1
Me5	mesencephalic trigeminal nucleus
MGE	medial ganglionic eminence
MZ	mantle zone
Nc	nucleus
Neurog1/2	neurogenin1/2
NICD	notch intracellular domain
NTZ	nuclear transitory zone
OB	olfactory bulb
OPC	oligodendrocyte precursor cell
P	postnatal day
P	Purkinje cells
PBS	phosphate buffered saline
PCL	pyramidal cell layer
PCR	polymerase chain reaction
PCRtA	parvicellular reticular nucleus
PDGFRα	platelet derived growth factor receptor
Pir	piriform cortex
pMN	motoneuron progenitor
P_N	neuronal progenitor
Pn	pons

PN	pontine nuclei
PNS	peripheral nervous system
POA	preoptic area
Pr5	the primary sensory trigeminal nucleus
PT	pretectal nucleus
Ptf1a	pancreatic transcription factor 1a
RL	rhombic lip
RMS	rostral migratory stream
Rt	reticular thalamic nucleus
Rtgn	reticulotegmental nucleus
Sc	superior colliculus
SCL	stem cell leukemia
SEM	scanning electron micrograph
SGZ	subgranular zone
Shh	sonic hedgehog
SNR	substantia nigra (reticular part)
Sox	SRY-box containing gene
Sp5	spinal trigeminal nucleus
Spt	septum
Str	striatum
SVZ	subventricular zone
Tg	transgenic
TH	thalamus
TH	tyrosine hydroxylase
TM	tamoxifen
URL	upper rhombic lip
UTR	untranslated region
VM	medial vestibular nucleus
Vsp	spinal vestibular nucleus
vVZ	ventral ventircular zone
VZ	ventricular zone
wm	white matter
YFP	yellow fluorescent protein

CHAPTER ONE

INTRODUCTION

The central nervous system, the brain and spinal cord, is perhaps the most complex object in the universe. Its functional architecture operates our mind and behavior controlling how we perceive, feel, think, act and remember. Understanding principles underlying brain function is fundamental not only in biological science but also in numerous disciplines such as psychology and philosophy. The complicated and elaborate neural network in the brain is dependent on the interconnections between tremendously diverse neural cell types. For example, one hundred billion neurons in the human brain are individual in their morphology, position, neurotransmitter, and the synaptic connection (Kandel et al., 1991). Moreover, different types of glia contribute to the integrity of neural computation with unique functions.

These diverse neural cell types are generated from a relatively simple embryonic structure called the neural tube. Initially, the three main layers of the embryo, the endoderm, mesoderm, and ectoderm arise at the gastrulation stage during embryo development. Inductive signals such as the bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) from the notochord or flanking ectoderm help form and pattern the neural tube (Tanabe et al., 1995; Tanabe and Jessell, 1996). Before the initiation of neurogenesis, the neural tube is composed of a pseudostratified neuroepithelium, a single layer of cells. These neuroepithelium cells presumably serve as the neural stem cells giving rise to all neurons and glia in the central nervous system (CNS) (Fig. 1.1A).

Neural stem cells are uncommitted multipotent progenitors with the ability to self-renew (Gotz and Huttner, 2005). By asymmetric and symmetric cell divisions, neural stem cells and progenitors first generate neurons and then glia during embryogenesis. In early symmetric divisions, the neural stem cells expand their numbers (Fig. 1.1B). Asymmetric cell divisions result in the neural stem cells generating a daughter stem cell and a lineage restricted progenitor or neuron. Lineage restricted progenitors divide symmetrically to give rise to two progenitors or two neurons. Time-lapse imaging in rat cortical slices described these distinct division patterns of stem or progenitor cells (Miyata et al., 2004; Noctor et al., 2004). In addition, clonal analysis of cortical progenitors in vitro revealed that each neuron and glia is generated with distinct cell division modes and orders (Qian et al., 2000; Shen et al., 2006). Not only cell division pattern but also the cell biological properties of early stem or progenitor cells have been described (Gotz and Huttner, 2005). Initially, the neuroepithelial cells are polarized along the apical-basal axis of the neural tube and express epithelial cell markers such as Nestin or Prominin-1. As neurogenesis begins, some neural stem cells derived from the neuroepithelium give rise to radial glia. Radial glia exhibit astroglial properties such as their ultrastructural character - glycogen granules, and astrocyte specific molecules such as GLAST (astrocyte-specific glutamate transporter), GFAP (glial fibrillary acidic protein), and BLBP (brain-lipid-binding protein) - while retaining some neuroepithelial features. In contrast to the multipotent neural stem cells from neuroepithelial cells, radial glia seem to be restricted to either neurons or glia (Malatesta et al., 2003; Anthony et al., 2004).

Generation of diverse neural cell types: spatial versus temporal cues

The early neural precursors appear uniform. They are located in the ventricular zone (VZ), a germinal zone proximal to the ventricle, of the neural tube. As the cells start to differentiate, the proliferating progenitors migrate laterally into the

mantle zone, exit the cell cycle to become postmitotic and differentiate to neurons. However, throughout the course of development, these homogenous progenitors give rise to tremendously diverse neural cell types: neurons, oligodendrocytes and astrocytes. Specific neural cell types are determined based on where and when the cells are generated. Here, using the spinal cord and the retina as models, I describe the principles underlying how neuronal diversity is achieved by positional and temporal information.

Spatial patterning and neurogenesis in the spinal neural tube

The molecular machinery to set up distinct neuronal cell types in the spinal neural tube is initiated by morphogens as gradient signals (Jessell, 2000). Secreted from organizing centers such as roof plate, the notochord and floor plate, the morphogens provide positional information (Tanabe and Jessell, 1996). Morphogens form a concentration gradient that partitions the developing neural tube into discrete subdomains. Thus, distinct threshold concentrations of morphogens at certain positions are converted into differential cell intrinsic profiles such as distinct homeodomain transcription factors in the progenitor domain (Fig. 1.2). These initial patterns are subsequently sharpened by cross-repressive interactions between transcription factors in neighboring progenitor domains. Combinatorial codes of transcription factors in the ventricular zone of a given subdomain instruct specific cell fates. For example, sonic hedgehog (Shh) plays key roles in controlling the specification of neural cell types in the ventral neural tube (Ericson et al., 1997; Briscoe et al., 2000). Shh secreted from the notochord and floor plate induces specific homeodomain protein codes for specific motoneuron or ventral interneurons in a concentration dependent manner along the dorsoventral axis of neural tube. In the dorsal region of the neural tube, the roof plate serves as an organizing center secreting extracellular proteins such as BMPs, FGFs or Wnts (Lee and Jessell, 1999; Lee et al., 2000). Gradients of

these factors are thought to form and will set up the dorsal progenitor subdomains for distinct interneuron populations. As a consequence of patterning, each progenitor domain expresses different combinations of transcription factors Pax3, Pax6, Pax7, Dbx2, Irx3, Nkx2.2 and Nkx6.1, functioning as a spatial code for specific neuronal fates. It is of note that homeodomain transcription factors are particularly important in the ventral neural tube patterning, whereas bHLH transcription factors have specification properties in the dorsal neural tube (Jessell, 2000; Gowan et al., 2001).

Different classes of neurons are generated not only along the dorsoventral axis but also the rostrocaudal axis of the neural tube (Dasen, 2009). For example, all motoneurons are generated from pMN domain along the dorsoventral axis of the neural tube. However, depending on their specific position along the rostrocaudal axis, the motoneurons obtain distinct columnar subtype identity exhibiting a specific connectivity with target muscles in the limb. FGF signaling from the paraxial mesoderm establish Hox-c protein gradients along the rostrocaudal spinal cord (Dasen et al., 2003) (Fig. 1.2B). Differential Hox-c profiles and activity impose positional identity on motoneurons along the rostrocaudal axis of the spinal cord (Dasen et al., 2003).

Unlike diverse types of neurons, the subtypes of oligodendrocytes and astrocytes are not well known or characterized. Not surprisingly, the molecular mechanism controlling the glia subtype diversity also remains poorly understood. However, there is accumulating evidence that different glia subpopulations seem to be generated from distinct progenitor domains. Oligodendrocytes had been thought to be generated solely from a ventral domain (pMN) in the neural tube (Richardson et al., 2006). Recent studies identified oligodendrocytes that were generated from dorsal regions of late-stage embryos (Cai et al., 2005; Fogarty et al., 2005; Vallstedt et al., 2005). Interestingly, the generation of this dorsal oligodendrocyte subpopulation seem to be controlled by distinct genetic programs

relative to the ventral population, suggesting they may be functionally distinct oligodendrocytes. Moreover, astrocytes are specified by positionally distinct progenitors expressing identified homeodomain codes in the ventricular zone of spinal neural tube (Hochstim et al., 2008). Thus, the fundamental principle for patterning neuronal cell type diversity may also be applied to glial cell type diversity, too. However, whether these glia with distinct origins lead to functionally different glia subtypes remains to be determined.

Temporal generation of neural cell types in the retina

In contrast to distinct progenitor domains patterned by spatial cues in the developing spinal cord, a common multipotent progenitor population in the inner layer of the optic cup give rises to all cell types in the retina (Marquardt and Gruss, 2002). Distinct retinal cell types are generated sequentially from this common progenitor pool in a defined temporal order. Retrovirus mediated cell lineage tracing and cell birthdating studies revealed that the timing of cell differentiation is critical to determine each cell fate (Turner and Cepko, 1987). In the beginning, neural progenitor cells expand their numbers by symmetric cell divisions as has been described in the cortex. As retinogenesis proceeds, retinal ganglion cells, horizontal cells, cone-photoreceptors, amacrine cells, rod-photoreceptors, bipolar cells and Müller glia are generated in chronological but overlapping phases. Cell extrinsic signals and intrinsic factors such as bHLH transcription factors are known to control the competency of the progenitors and the specification of certain cell types over time (Hatakeyama and Kageyama, 2004).

The spinal neural tube and the retina were chosen to emphasize the importance of positional and temporal information, respectively, for regulating neural cell type diversity. However, to construct the elaborate cytoarchitecture of

each anatomical structure in the CNS, both spatial and temporal information must be integrated and interpreted coordinately rather than being used separately (Pearson and Doe, 2004; Kutejova et al., 2009). In the spinal cord, a single progenitor domain (pMN) defined by a transcription factor, Olig2, sequentially generates motor neurons, interneurons and oligodendrocytes (Zhou and Anderson, 2002; Wu et al., 2006). This is a good example that the mechanisms regulating cell competency or lineage restriction over time as seen in the retina also govern spinal cord cell type diversification. In the telencephalon, two sets of bHLH transcription factors (Neurog1/2 and Ascl1) are expressed in the dorsal and ventral germinal zones, respectively, in a mutually exclusive manner (Parras et al., 2002). Each domain gives rise to distinct neural cell types: dorsal progenitors give rise to excitatory neurons including pyramidal neurons in the neocortex, whereas the ventral progenitors give rise to inhibitory interneurons in the cortex and the other basal ganglionic structures (Parras et al., 2002). Thus, dorsoventral identity of progenitor domains determines the neurotransmitter phenotype of neurons. Temporal cues such as cell birthdates are essential to determine their subtype identity such as their position in cortical layers (Olson and Walsh, 2002; Rakic, 2009). In the ventricular zone of the dorsal telencephalon, neurons born at early stages migrate to the deep layers of the cortex, whereas neurons born at late stages migrate to the superficial layers. Recent lineage tracing studies show the timing of differentiation is also important for determining the layer identity of specific interneuron populations from progenitor domains in the ventral telencephalon (Butt et al., 2005). Therefore, the precise coordination of temporal and spatial cues is a fundamental mechanism governing neural cell type diversity.

Basic helix-loop-helix factors (bHLHs) in neurodevelopment

With the advance of molecular biology, considerable effort has been made to elucidate the genetic and molecular mechanisms regulating neural cell type

diversity. bHLH transcription factors are essential cell intrinsic factors controlling multiple mechanisms during neurogenesis and gliogenesis (Bertrand et al., 2002; Ross et al., 2003). The bHLH transcription factors are defined by a shared structural motif: a basic region and two α-helices connected by a loop. The helix regions are required for dimerization with E-proteins, more broadly expressed bHLH proteins such as Tcf4, E12, E47 or HEB, and the basic domain is required for DNA binding to activate or repress downstream gene transcription (Murre et al., 1989; Bertrand et al., 2002).

Genetic studies in *Drosophila* discovered 'proneural' genes of the achaete-scute complex encoding bHLH transcription factors (Garcia-Bellido, 1979). The proneural bHLH factors are necessary and sufficient to initiate the neuronal lineage program in the ectoderm. The first mammalian homolog of the achaete-scute complex genes was identified in 1990 using degenerative oligonucletide primers and polymerase chain reaction (PCR) from RNA from a sympathoadrenal progenitor cell line (Johnson et al., 1990). This gene was named Mash1 (mammalian homolog of achaete-scute complex 1), but now is called Ascl1 (achaete-scute homolog 1). Other vertebrate neural bHLH factors including Atoh1 (previously Math1), Neurog1/2 (Neurogenin1/2), Ptf1a (pancreatic transcription factor 1a) and Olig1/2 were identified later (Ma et al., 1996; Ben-Arie et al., 1997; Lu et al., 2000; Zhou et al., 2000; Glasgow et al., 2005). Temporally, neural bHLH transcription factors fall into multiple subclasses were some such as those mentioned above are largely restricted to proliferating or early differentiating progenitors. Spatially, these bHLH factors are expressed in discrete regions of developing CNS with largely non-overlapping manner (Gowan et al., 2001; Helms and Johnson, 2003). Other classes of bHLH transcription factors such as NeuroD or NeuroM (also known as Atoh3) are expressed and function in postmitotic precursors (Ross et al., 2003). To focus on the functions of bHLHs for

neural cell type diversity, the discussion below will be restricted to bHLH factors expressed in the VZ.

Functions of bHLH transcription factors for discrete neural cell types: differentiation and subtype specification

Mutant mice null for each bHLH factor and several over expression paradigms demonstrated the essential functions of each bHLH gene in distinct cell types in both CNS and PNS (peripheral nervous system) development. Ascl1 is required for specific subsets of neurons in dorsal spinal cord and telencephalon, mesencephalon, hindbrain, olfactory sensory epithelium, and neurons in the autonomic nervous system in the periphery (Guillemot et al., 1993; Blaugrund et al., 1996; Cau et al., 1997; Hirsch et al., 1998; Casarosa et al., 1999; Akagi et al., 2004; Miyoshi et al., 2004; Pattyn et al., 2004; Helms et al., 2005). In addition, Ascl1 is required for subpopulations of oligodendrocyte development in telencephalon and spinal cord (Parras et al., 2007; Sugimori et al., 2007; Sugimori et al., 2008). Atoh1 is essential for commissural interneurons in the dorsal spinal cord, external granule cells in the cerebellum, discrete glutamatergic neurons in certain brain stem nuclei and sensory epithelium of the inner ear (Ben-Arie et al., 1997; Gowan et al., 2001; Helms et al., 2001; Machold and Fishell, 2005). Neurog1 and Neurog2 are required for different subsets of dorsal root and cranial sensory ganglia, dorsal interneurons in the spinal cord, glutamatergic neurons in the cortex, and dopaminergic neurons in the midbrain (Ma et al., 1996; Sommer et al., 1996; Gowan et al., 2001; Parras et al., 2002; Kele et al., 2006). Ptf1a is essential for GABAergic neurons in the dorsal spinal cord, cerebellum, and retina (Glasgow et al., 2005; Hoshino et al., 2005; Fujitani et al., 2006).

bHLH transcription factors exert their functions in cell differentiation and subtype specification. Their functions have been studied extensively using mouse mutants null for each bHLH gene and overexpression paradigms such as in ovo

electroporation or neural cell cultures. In the ventricular zone of mouse embryos null for *Ascl1*, the progenitors continue to proliferate aberrantly (Casarosa et al., 1999; Horton et al., 1999; Wildner et al., 2006; Battiste et al., 2007). Conversely, overexpression of bHLH factors in neural cell cultures or chick neural tube and retina force the cells to exit the cell cycle, move laterally out of the ventricular zone, and initiate the expression of neuronal specific genes such as Tuj1 or NeuN (Farah et al., 2000; Nakada et al., 2004; Helms et al., 2005; Kriks et al., 2005). Neural bHLH factors may promote a neuronal differentiation program through at least two mechanisms. First, Ascl1 and Neurog1 have been suggested to couple neuronal differentiation to withdrawal from the cell cycle, possibly by activating the expression of p27 (Kip1), a cyclin-dependent kinase inhibitor (Farah et al., 2000). Second, Ascl1 and Neurog1 can induce cascades of neuronal differentiation genes such as *NeuroD* or *NeuroM* (Fode et al., 1998; Cau et al., 2002). The cell differentiation function of proneural bHLH factors is known not only to promote neuronal differentiation but also to inhibit glial fates. For example, in rat cortical progenitor cultures, Neurog1 promotes neurogenesis and inhibits astrocyte formation through the JAK-STAT pathway (Sun et al., 2001). In the absence of *Ascl1* and *Atoh3*, the generation of Müller glia is enhanced in addition to the inhibition of neuronal differentiation in the retina (Tomita et al., 2000). Since neurons and glia should be generated sequentially from multipotent progenitors, the dual roles of proneural bHLH ensure the correct coordination of neurogenesis and gliogenesis without depleting progenitor pools.

Neural bHLH transcription factors are also important for neuronal subtype specification. Mouse mutants and in ovo overexpression studies demonstrated that Ascl1, Atoh1, and Neurog1/2 specify neuronal subtypes in the dorsal neural tube, the telencephalon and the peripheral nervous system (Gowan et al., 2001; Parras et al., 2002; Nakada et al., 2004; Helms et al., 2005). For example, Ascl1 is essential for dI3, dI5 and dIL^{A} neurons in the dorsal spinal cord whereas Atoh1

and Neurog1 are for dI1 and dI2 neurons respectively. Mutant mice where Neurog2 replaced Ascl1 or vice versa, demonstrated that Neurog2 and Ascl1 have distinct roles in the specification of glutamatergic and GABAergic neurons in dorsal and ventral telencephalon, respectively (Parras et al., 2002).

Identifying transcriptional targets of bHLH factors is fundamental to provide mechanistic insights of bHLH functions in neurogenesis. Proneural bHLH proteins form heterodimers with E-proteins to bind a DNA sequence called the E-box (CANNTG) (Murre et al., 1989). However, it has been difficult to identify and validate direct in vivo downstream targets just based on the degenerate 6 base pair DNA motif. With advanced technology using chromatin immunoprecipitation, microarray and bioinformatics, several in vivo transcriptional targets of bHLHs are beginning to be identified. Rnd2 expression, a small GTPase protein, is directly controlled by Neurog2 in the telecenphalon, and it regulates neuronal migration (Heng et al., 2008). Notch ligands such as *Dll1* and *Dll3* are transcriptional targets of Ascl1. Notably, Ascl1 interacts with Brn1/2 or Neurog2 on the promoter of *Dll1* and *Dll3*, providing more specific DNA binding motifs to screen their candidate target genes in the future (Castro et al., 2006; Henke et al., 2009). Ptf1a trimer complex with E-protein and RBPj regulates *Neurog2* in the dorsal neural tube and the cerebellum (Hori et al., 2008). It is important to note that a bHLH transcription factor may regulate shared or distinct downstream targets for different functions. In addition, a downstream target can be regulated by multiple transcription factors at different levels. Identification of the full repertoire of shared or distinct transcriptional targets of each bHLH transcription factor will shed light to understand their roles in the vertebrate CNS development at molecular level.

bHLH factors as inhibitors of differentiation

Two additional classes of bHLH (or HLH) transcription factors play important roles in inhibition of neuronal differentiation. These include the Hes family, mammalian homologues of *Drosophila* hairy and Enhancer of split gene, and Ids, inhibitors of differentiation. Most Hes factors act as transcriptional repressors. Hes1, Hes3, and Hes5 are expressed in undifferentiated neural stem cells of the ventricular zone (Kageyama et al., 2008a). In mice null for *Hes* genes, proneural bHLH factors such as Ascl1 and Neurog2 are upregulated and neuronal differentiation is accelerated (Ishibashi et al., 1995; Chen et al., 1997). As a consequence, the neural stem cell populations get depleted. Conversely, overexpression studies showed that Hes1 or Hes5 inhibits neurogenesis (Ohtsuka et al., 1999; Cau et al., 2000). Hes proteins bind the enhancer regions of proneural genes such as *Ascl1* and repress their transcription (Ishibashi et al., 1995; Chen et al., 1997). Another important feature of Hes proteins is their oscillatory expression that is dependent on negative autoregulation (Kageyama et al., 2007). For example, Hes1 represses its own transcription by binding its promoter. Since Hes1 has a short half-life, this negative feedback loop becomes oscillatory in a cell autonomous manner. Although the oscillation of Hes1 expression is important to regulate the timing of cellular events such as somite segmentation, its precise function in nervous system development remains unclear.

Ids provide another level of control of neuronal differentiation. Both Ids and Hes inhibit neuronal differentiation, but in a different manner. Ids cannot function as transcription factors since they lack the basic DNA binding domain (Norton, 2000). Rather, Ids sequester E-proteins from heterodimerizing with proneural bHLH factors by forming Id/E heterodimers . Ids are expressed in an overlapping manner with Hes genes prior to the expression of proneural genes. Overexpression of Id in the chick hindbrain inhibits proneural genes but increase Hes1 expression, resulting in the inhibition of neurogenesis (Bai et al., 2007). Additional complexities are evident from a recent study that showed Id can

interact directly with Hes1 to suppress Hes1's negative feedback (Bai et al., 2007).

The Notch signaling pathway

The Notch signaling pathway is the central mechanism that controls cell fate choices throughout neural development. Notch signaling and its interplay with proneural bHLH transcription factors control binary cell fate choices by setting up the unequal distribution of Notch receptors, ligands, and proneural bHLH proteins among otherwise homogenous progenitor cells (Yoon and Gaiano, 2005; Louvi and Artavanis-Tsakonas, 2006).

Notch signaling controls neural differentiation through lateral inhibition by controlling the balance between proneural and Hes transcription factors (Yoon and Gaiano, 2005; Louvi and Artavanis-Tsakonas, 2006). At early stages, neural progenitors are equivalent with similar levels of Notch receptor and ligand expressed on their surface (Fig. 1.3 and 4.1). Likely due to stochastic variation, a cell starts to express higher levels of a Notch ligand such as Dll1 (deltalike1). Upon activation of Notch receptor by Dll1 in the neighboring cell, the Notch intracellular domain (NICD) is translocated from the plasma membrane into the nucleus. In the nucleus, NICD converts RBPj (also known as CBF1) from a repressor to an activator by forming a transactivator complex with Maml (mastermind-like). This transcription complex upregulates expression of anti-neurogenic bHLH genes such as *Hes1* or *Hes5*. Hes1 and Hes5 directly suppress the transcription of proneural genes such as *Ascl1* or *Neurog1/2*. Since *Dll1* is the downstream target of Ascl1 or Neurog1/2, Notch ligand expression will be lowered in that cell. Thus, the imbalance of Notch signaling between two initially equal adjacent cells will be amplified. As a consequence, a cell having higher levels of Dll1 and proneural bHLH factor expression will initiate differentiation

whereas a cell having higher Notch activation and *Hes* gene expression will remain as a progenitor.

Recently, this classic view of Notch signaling was updated by including oscillation of gene expression into the model (Kageyama et al., 2008b). Real time imaging of slice cultures from the dorsal telencephalon showed that Hes1 expression in a cell is indeed dynamically upregulated and downregulated with a 2-3 hour periodicity (Shimojo et al., 2008). Dll1 and Neurog2 also oscillate but with an inverse wave (Shimojo et al., 2008). Thus, Neurog2 and Dll1 expression does not necessarily predict that a cell will differentiate into a neuron. In this model, the mechanism that selects the differentiating cell still remains unknown. One possibility is that cyclic expression of proneural factors result in the accumulation of downstream factors; factors that may regulate cell cycle exit or neuronal differentiation. Although the biological significance of the oscillation model remains to be studied, the oscillatory expression of Notch players may maintain the cells more alert for responding to differential cell signaling pathways important for cell differentiation.

bHLH factors in gliogenesis

bHLH transcription factors also play important roles in oligodendrocyte specification. Olig1 or Olig2 are expressed in the ventral neural tube in the pMN domain, or in the progenitor domain of ventral telencephalon where oligodendrocyte precursors emerge (Lu et al., 2000; Zhou et al., 2000; Zhou et al., 2001). Unlike proneural bHLH factors, the expression of Olig1 and Olig2 are sustained into mature oligodendrocytes. Studies using *Olig* null mice demonstrated that Olig1 and Olig2 are essential for oligodendrocyte cell fate determination and maturation (Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). Olig2 is also required to generate motoneurons in the spinal cord, GABAergic interneurons in the ventral telencephalon, and astrocytes in the

cortex (Lu et al., 2002; Cai et al., 2007). How Olig2 functions in these different cell types remains to be uncovered.

bHLH transcription factors are also involved in generating astrocytes. In the cortex and the retina, Hes genes are expressed in differentiating astrocytes or Müller glia. Overexpression of Hes1 or Hes5 led to differentiation to astrocytes at the expense of neurogenesis in these systems (Ohtsuka et al., 1999; Ohtsuka et al., 2001). Conversely, in the mouse retina null for *Hes1*, Müller glia cell numbers are greatly decreased (Takatsuka et al., 2004). In contrast, proneural genes suppress astrocyte specification. Mice null for both *Neurog2* and *Ascl1* showed premature and excessive astrogenesis in cortical development (Nieto et al., 2001). Taken together, mechanisms for generating astrocytes seem to work by an interplay between two classes of bHLH factors, likely through their interplay with Notch signaling. Interestingly, Stem cell leukemia (SCL), another bHLH transcription factor, was shown to be necessary and sufficient for astrocyte specification at least in specific regions of the developing neural tube (Muroyama et al., 2005). This suggests a more 'active' transcriptional process for astrogenesis exists than was previously appreciated.

Adult neurogenesis and gliogenesis

It is now widely accepted that new neurons continue to be generated from adult neural stem cells in restricted regions of the adult brain throughout life (Gage, 2000; Alvarez-Buylla and Lim, 2004). This new view is contrary to the long-held dogma in modern neuroscience that all neurons in the adult CNS are terminally differentiated. Integration of new neurons in the adult brain adds another level of plasticity into the neural network, implicating adult neurogenesis has important roles in brain function. Moreover, understanding adult neurogenesis provides an exciting potential to develop new treatments for neurodegenerative diseases such as Alzheimer's or Parkinson's disease, or other brain injuries.

Adult neural stem cells are present in two restricted neurogenic niches of the forebrain: the subventricular zone (SVZ) around the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Doetsch and Hen, 2005) (Fig. 1.4A-B). Similar to embryonic neural stem cells, the self-renewing adult neural stem cells have a distinct radial glia like morphology and express astrocytic/radial glia markers such as GFAP, BLBP, Nestin, or GLAST. In the SVZ, GFAP expressing B cells are the only self-renewable stem-like cells that divide asymmetrically (Doetsch et al., 1999; Doetsch, 2003; Alvarez-Buylla and Lim, 2004). B cells differentiate into transit amplifying C cells. C cells are mitotically active and express early neural lineage markers such as Sox2 or Ascl1, but are negative for astrocytic markers. C cells further mature into the neuroblasts called A cells. A cells migrate along the rostral migratory stream (RMS) towards the olfactory bulb where they differentiate. In the granule cell layer and the periglomerular layer of the olfactory bulb, adult generated neurons mature into GABAergic interneurons or glutamatergic juxtaglomerular neurons (Alvarez-Buylla and Lim, 2004; Brill et al., 2009). In the adult hippocampus, the $GFAP^+$ type-1 cell is the putative stem-like cell which can self-renew in vitro, comparable to the astrocyte-like B cells (Seri et al., 2001; Seri et al., 2004) (Fig. 1.4C). Type-1 cells differentiate into the actively proliferating type-2 and -3 cells sequentially (Kempermann et al., 2004; Steiner et al., 2006). Type-2 and -3 cells have a distinct morphology from type-1 cells and express the neural lineage markers Ascl1, NeuroD1 and Doublecortin (Dcx) sequentially. Unlike the different subtypes of newly generated neurons in the OB, the adult generated neurons in the hippocampal dentate gyrus become only excitatory granule neurons (Doetsch and Hen, 2005).

An important feature of the microenvironments in the adult neurogenic niches is the presence of extracellular or trophic signals permissive and instructive for the stem cell maintenance and differentiation (Alvarez-Buylla and Lim, 2004;

Johnson et al., 2009). The wide variety of signaling molecules including developmental morphogens have been studied extensively for adult neurogenesis in vitro and in vivo. Canonical Wnt signaling is active in the SGZ of adult hippocampal dentate gyrus (Lie et al., 2005). Overexpression of Wnt3 increases proliferation of the adult progenitors and production of Dcx^+ neuroblasts whereas Wnt inhibitors or dominant negative variants have the opposite effect in the adult hippocampus. Adult neural stem cells are also known to respond to BMP or Shh signaling (Alvarez-Buylla and Lim, 2004). In contrast to their spatially separable expression in dorsal and ventral neural tube, respectively, both BMP and Shh are intermixed in the adult SVZ. Molecular fate mapping studies using *Gli1-CreER*^{T2}, a downstream effector of Shh, demonstrated that $GFAP^+$ stem cells respond to the Shh signaling and continue to generate neurons throughout life (Ahn and Joyner, 2005). Interestingly, Shh and BMP have opposite functions: proliferation of progenitor cells is promoted by Shh signaling, but inhibited by BMP signaling (Alvarez-Buylla and Lim, 2004). Therefore, the balance between Shh and BMP signaling must be important for maintaining the stem cell populations.

Notch components such as Notch1, Hes, and Ascl1 are also present in the adult brain in regions undergoing neurogenesis (Breunig et al., 2007). Not surprisingly, Notch signaling pathways appear to have pleiotropic roles in adult neurogenesis. The increase or decrease in the number of proliferating cells in experimental paradigms with overexpressing NICD or conditionally ablating *Notch1,* respectively, suggests Notch regulates the maintenance of progenitor cells in the adult neurogenic niche (Breunig et al., 2007). Accordingly, *Notch1* loss leads to an increase in the number of the new born neurons at the expense of progenitor cells, whereas overexpression of NICD results in more $Sox2^+$ or $GFAP^+$ progenitors with fewer Dcx^+ neuroblasts. In addition, even dendrite morphology of the newly generated neurons is affected by genetic manipulation

of Notch signaling in adult brain, suggesting Notch has multiple functions in these lineages (Breunig et al., 2007).

Transcriptional mechanisms controlling neuronal differentiation and specification in adult neurogenesis are beginning to be clarified. Distinct stages of adult neural stem cells as they progress along their lineage development to mature neurons are now characterized by the presence of specific proteins, often transcription factors. Sox1-3 mark early progenitor populations including the stem-like cells (Wang et al., 2006; Lefebvre et al., 2007). Ascl1 transiently labels type-2a cells and a minor population of neural stem like cells (Kim et al., 2007). NeuroD1, Neurog2, Tbr2 labels the intermediate stage progenitors such as type-2b, type-3, or neuroblast (Steiner et al., 2006; Ozen et al., 2007; Hodge et al., 2008). Dlx2, Pax6, and Olig2 are also expressed in the subpopulations of neural progenitors (Doetsch et al., 2002; Hack et al., 2005). Since the mice null for these transcription factors are either lethal at birth or have severe developmental defects, it has been challenging to investigate their functions during adult neurogenesis. Recently, the conditional deletion of *NeuroD1* in the adult hippocampal progenitors was examined and shown to lead to neuronal differentiation and survival defects (Gao et al., 2009). Increased or decreased levels of transcription factors such as Pax6, Olig2 or Sp8 in progenitors alters their neuronal subtypes to GABAergic or dopaminergic in adult olfactory bulb neurogenesis (Hack et al., 2005; Waclaw et al., 2006). In addition, the overexpression of Ascl1 in adult hippocampal progenitors redirects their fates to the oligodendrocyte lineages (Jessberger et al., 2008). The functions of other bHLH transcription factors in the adult brain remain to be investigated. It would be important and intriguing to understand how closely the transcriptional programs used in adult neurogenesis mimic those controlling embryonic neurogenesis, as it would provide the molecular strategies to manipulate the neural stem cell potentials for therapeutic use.

Oligodendrocytes and astrocytes are also generated in the adult brain. Adult gliogenesis appears to be associated mainly with glial restricted precursors such as OPCs (oligodendrocyte precursor cells) or reactive astrocytes rather than neural stem cells in the SGZ or the SVZ (Ridet et al., 1997; Polito and Reynolds, 2005). NG2 expressing OPCs are spread broadly throughout the brain (Polito and Reynolds, 2005). Responding to demyelinating brain insults or less actively in normal condition, this dividing precursor population generates new oligodendrocytes. $GFAP^{+}/Vimentin^{+}/S100\beta^{+}$ reactive astrocytes also proliferate responding to pathological brain injury or post-lesion recovery (Ridet et al., 1997). Adult neural stem cells are also known to generate oligodendrocytes or terminally differentiated astrocytes (Menn et al., 2006; Suh et al., 2007). A small subpopulation of transit amplifying C cells express Olig2, an oligodendrocyte lineage marker (Menn et al., 2006). Retrovirus mediated lineage tracing showed that these cells give rise to myelinating oligodendrocytes in the corpus callosum or the fimbria. In the adult hippocampus, Sox2 expressing adult progenitors give rise to astrocytes as well as neurons (Suh et al., 2007). However, the clear lineage relationship among adult generated neurons, oligodendrocytes and astrocytes remains to be delineated. Cell intrinsic mechanisms regulating adult gliogenesis are poorly understood. As Olig1, bHLH transcription factor, is required to repair demyelinated lesions in the adult brain (Arnett et al., 2004), it is plausible that transcription factors regulating embryonic glial development also participate in adult gliogenesis.

Thesis rationale and goals

The research presented in this thesis is aimed towards gaining a better understanding of in vivo lineages of diverse neurons and glia in the CNS. Specifically, I have been interested in how Ascl1 contributes to neural cell diversity in vertebrate development. To tackle this fundamental question, I have

generated diverse tools used to perform lineage analysis of Ascl1 expressing progenitors from the embryo to the adult to provide a framework to address the molecular control of nervous system formation. In chapter two, the contribution of Ascl1 to many diverse cell types in embryonic brain development will be emphasized. In chapter three, using two different Cre expressing mouse models marking Ascl1 lineage cells at temporally distinct stages, I propose similar lineage models for neurogenesis and gliogenesis in the embryonic spinal cord. In addition, the distinct functions of Ascl1 in neurogenesis and gliogenesis were assessed using *Ascl1* conditional mutant mice. The functional importance of Nicastrin, as it pertains to Ascl1 function and regulation of Notch signaling in gliogenesis will be discussed in chapter four. Lastly, in chapter five, I will characterize the identity and dynamics of Ascl1 expressing progenitors during adult neurogenesis and gliogenesis.

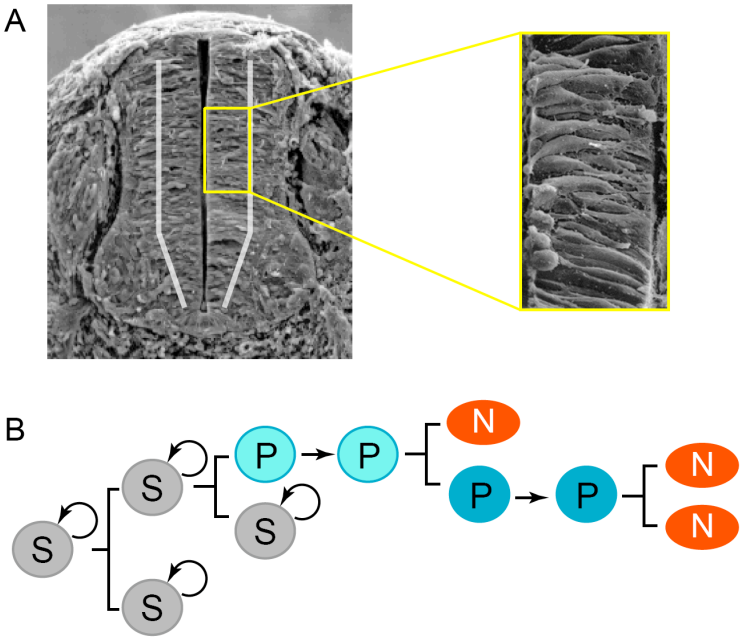

Figure 1.1. Neural tube and possible lineage relationships during neurogenesis. (A) Transverse section of E11 mouse neural tube by scanning electron micrographs (SEM). In the magnified panel (yellow), neural stem cells have distinct elongated morphology along the apical-basal axis. (B) Simplified lineage tree of neurogenesis. Neural stem cells undergo proliferative divisions by symmetric cell division. Then, they divide asymmetrically to generate one stem cell daughter and one non-stem cell progenitor. Non-stem cell progenitors generate one progenitor daughter with a more restricted potential and one neuron. The restricted progenitor divides symmetrically to generate two neurons. Abbr: N, neuron; P, progenitor; S, stem cell. Modified from Götz and Huttner, 2005 and http://www.med.unc.edu/embryo_images.

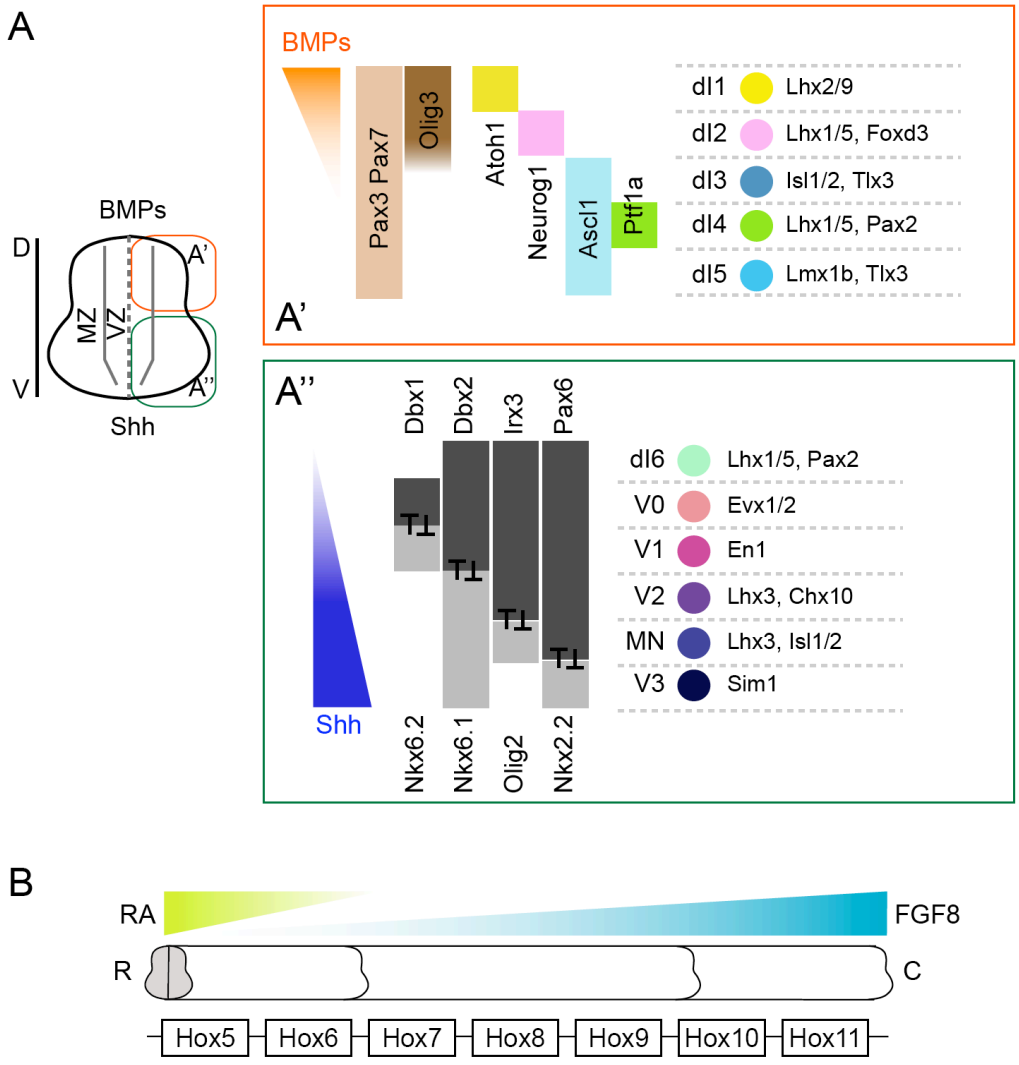

Figure 1.2. Spatial patterning of the developing spinal cord.
(A) Schematic drawing illustrates morphogens from the roof plate (BMP) and the floor plate (Shh) along the dorsoventral axis of the neural tube. (A') Dorsal patterning. The progenitor domains are defined by bHLH and homeodomain factors. dI1-3 neurons are dependent on BMP. (A'') Ventral patterning. The discrete progenitor domains are set up by cross-repressive interaction between patterning proteins regulated by Shh. In both dorsal and ventral neurons, a combinatorial transcription code determines their postmitotic identity. (B) Rostocaudal patterning of the spinal cord. Retinoic acid (RA) and FGF gradients induce differential Hox gene expressions. Modified from Briscoe et al., 2000, Helms and Johnson, 2003, Dasen, 2009.

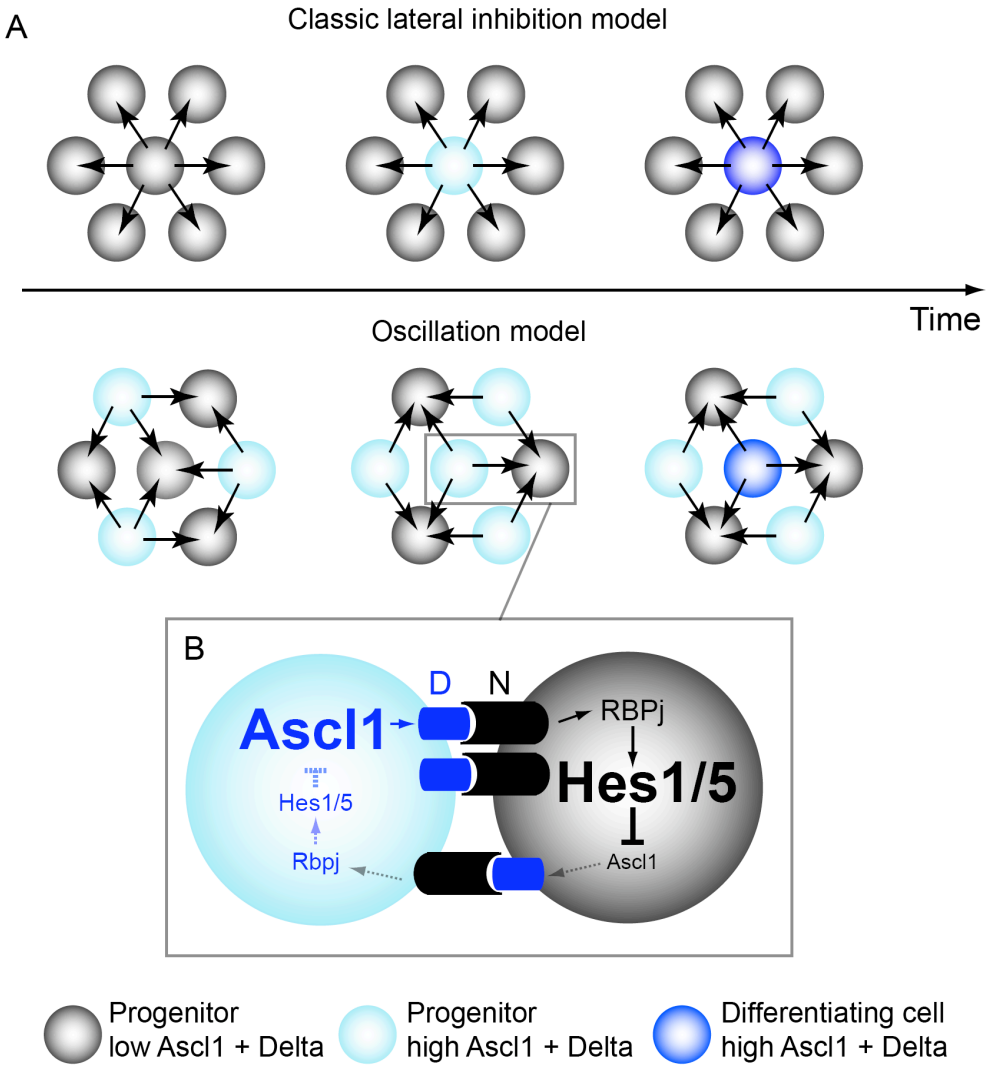

Figure 1.3. Classic versus oscillation models of lateral inhibition in Notch signaling pathway.
(A) In the classic lateral inhibition model, all progenitors are equivalent with similar levels of Notch ligand Delta and proneural proteins such as Ascl1. A subtle stochastic difference in Ascl1 and Delta expression between adjacent cells is amplified by lateral inhibition. The progenitor having highest level of Ascl1 and Delta expression differentiates into neurons, whereas the neighboring cells remain as progenitors. In the oscillation model, Ascl1, Delta and Hes1 oscillate during different phases of cell cycles, forming salt-and-pepper patterns. Thus, substantial accumulation of Ascl1 and Delta or

Hes1 is precluded by their oscillatory dynamics. This view emphasizes more dynamic reciprocal signaling between progenitors. (B) In the cell having higher level of proneural gene Ascl1, Delta expression is increased and activates Notch signaling in the neighboring cell. In the cell having Notch signaling activation, RBPj containing transcriptional activator upregulates Hes1 and Hes5 expression. In turn, the transcription of the proneural gene *Ascl1* is suppressed by Hes1 and Hes5. See also Figure 4.1 for detailed description of Notch signaling pathway. Abbr: D, Delta; N, Notch receptor. Modified from Kageyama et al., 2008.

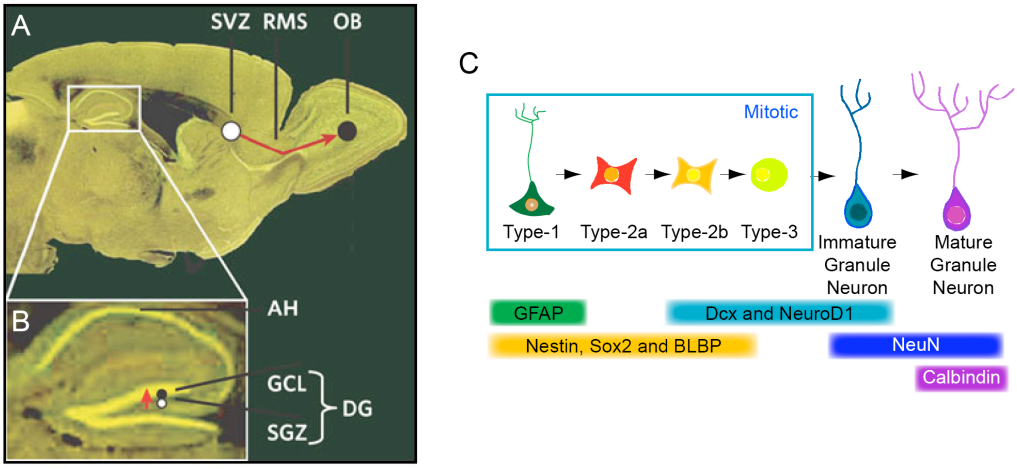

Figure 1.4. Two neurogenic niches in the adult brain.
(A) Transverse section of adult mouse brain. Stem cells in the subventricular zone (SVZ) around the lateral ventricle differentiate and migrate along rostral migratory stream (RMS) to become the neurons in the olfactory bulb (OB). (B) In the hippocampal formation, stem cells located in the subgranular zone (SGZ) in dentate gyrus (DG) give rise to the excitatory neurons in granule cell layer (GCL). (C) Adult neurogenesis lineage models in the hippocampus. Type-1 neural stem cells have astrocytic features and are marked by GFAP. Although these cells have proliferative capacity, they are much more slowly cycling than the Type-2 progenitor cells. Nestin, Sox2 and BLBP are also expressed in Type-1 cells but they persist into the Type-2 cell stages. NeuroD and Doublecortin (Dcx) appear in Type-2b, the later stage of Type-2 cells, and persist into post-mitotic but immature granule cell precursors. Finally, cells mature into NeuN/Calbindin granule cell neurons in the dentate gyrus. Abbr: AH, Ammon's horn of hippocampus. Adapted and modified from Greenberg and Jin, 2005 and Steiner et al., 2006.

CHAPTER TWO

Ascl1 lineage cells contribute to discrete cell populations in CNS architecture

INTRODUCTION

In the mammalian central nervous system (CNS), distinct types of neurons are assembled into elaborately interconnected circuits that process complex neural functions. To obtain this refined CNS architecture, diverse populations of neurons must be generated that migrate to specific positions in precise temporal order during embryogenesis. The molecular mechanisms regulating each developmental step in this process are not completely understood. Basic helix-loop-helix (bHLH) transcription factors play a central role in generating neuronal diversity by regulating subtype specification as well as differentiation (Bertrand et al., 2002). Ascl1 (previously Mash1) is a neural bHLH transcription factor restricted to proliferative zones in the developing brain and spinal cord in a spatially specific manner. Multiple studies of mouse embryos lacking Ascl1 suggest that Ascl1 is a neuronal differentiation factor required in diverse but specific neuronal subtypes in the developing central and peripheral nervous systems (Horton et al., 1999; Perez et al., 1999; Fode et al., 2000; Nakada et al., 2004; Pattyn et al., 2004; Helms et al., 2005). Neuronal lineages disrupted in the Ascl1 mutant include interneurons in dorsal spinal cord and telencephalon, both glutamatergic and GABAergic neurons in the mesencephalon, olfactory sensory epithelium, and neurons in the autonomic nervous system in the periphery.

Within a specific neural region, Ascl1 can also influence neuronal subtype specification (Fode et al., 2000; Parras et al., 2002; Nakada et al., 2004; Helms et al., 2005). In dorsal spinal cord, mouse embryos null for Ascl1 lose dI3 and dI5

neurons, whereas overexpression of Ascl1 in the chick neural tube leads to an increase in these specific neuronal populations (Nakada et al., 2004). In neural crest derivatives, Ascl1 is required for autonomic neurons but not sensory neurons (Perez et al., 1999). In the ventral spinal cord, Ascl1 functions in balance with Notch signaling at the choice point where the V2 interneuron population is split into two subpopulations, V2a and V2b (Del Barrio et al., 2007; Peng et al., 2007). Furthermore, Ascl1 is not restricted to neuronal lineages but is also present in progenitors to oligodendrocytes, but not astrocytes, in the spinal cord and adult brain (Battiste et al., 2007; Kim et al., 2007).

A complete fate map of Ascl1 derived lineages, particularly in the brain, has not been described largely due to the transient nature of Ascl1 expression that disappears as the cells exit the cell cycle and migrate extensively during development. This hurdle has been overcome by utilizing Cre recombinase in an in vivo genetic fate mapping strategy to define mature CNS regions receiving contribution from progenitor cells that have transiently expressed Ascl1. This strategy was used to demonstrate that in spinal cord development, Ascl1 progenitors from embryonic day 10.5 (E10.5)-E12.5 give rise to dorsal horn interneurons whereas after E15.5 Ascl1 progenitors give rise to oligodendrocytes (Battiste et al., 2007). In the adult brain, Ascl1 was also found to be present in neuronal progenitors in the dentate gyrus of the hippocampus and the rostral migratory stream in the forebrain, and in oligodendrocyte precursors in white and gray matter (Kim et al., 2007). Consistent with this lineage analysis, forced expression of Ascl1 in neural progenitor cultures biased differentiation of the cells to neurons and oligodendrocytes at the expense of astrocytes (Gokhan et al., 2005; Sugimori et al., 2007).

Here I use the in vivo genetic fate mapping strategy to identify brain regions containing neurons and oligodendrocytes originating from Ascl1 expressing progenitor cells from different embryonic stages. I find that Ascl1-

expressing cells are generated continuously throughout embryogenesis from as early as E9.5. As in the spinal cord, the Ascl1 lineage includes both neurons and oligodendrocytes, but not astrocytes. I demonstrate Ascl1 is present in neural progenitor cells in each major brain division. In the cerebellum, depending on the embryonic stage, Ascl1 defined lineages give rise to GABAergic neurons in deep cerebellar nuclei and Purkinje cells. Ascl1 lineages contribute to the trigeminal sensory system from the midbrain to the caudal medulla. Multiple telencephalic regions contain Ascl1 lineage cells including subsets of neurons in the striatum, olfactory bulb, amygdala and piriform cortex. Neurons in the neocortex arise from Ascl1 progenitors present at late embryonic stages. This study correlates the temporal and spatial origin of Ascl1 expressing progenitor cells and their final phenotypes throughout the brain. The diverse identity of the neurons is consistent with Ascl1 requiring additional molecular components for its specification function.

MATERIALS AND METHODS

Transgenic mice and Tamoxifen injection

Ascl1-GIC, Ascl1-CreER^{TM}, R26R-stop-lacZ, and *R26R-stop-YFP* mice have been previously described. Briefly, *Ascl1-GIC* and *Ascl1-CreER^{TM}* mice are BAC transgenic mice where GFP-IRES-Cre or CreER^{TM} replaces the Ascl1 coding region, respectively (Helms et al., 2005; Battiste et al., 2007). *R26R-stop-YFP (or lacZ)* mice are Cre recombinase reporter mice (Soriano, 1999; Srinivas et al., 2001).

Tamoxifen induction of Cre recombinase was accomplished by intraperitoneal injection of pregnant females at a given day post coitum (dpc) twice with 6 hr interval (noon and 6 pm) with 50-75 mg/kg tamoxifen (Sigma, T55648) in sunflower oil. Embryonic or adult brains were harvested at the times specified after tamoxifen treatment.

X-gal staining and immunofluorescence staining

For X-gal staining, whole embryos (E9.5-13.5) or dissected brains (E15.5-P3) were fixed by immersion in 4% formaldehyde for 1-2 hours at room temperature. After washing in phosphate buffer, the tissues were incubated overnight in X-gal staining solution (1mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2mM $MgCl_2$ in PBS and 0.02% NP-40). Tissues were washed in phosphate buffer, post-fixed with 4% formaldehyde overnight at 4 ^{o}C, and vibratome sectioned at 200 μm. Adult brains were dissected from the skull after animals were anesthetized with Avertin and perfused with 4% formaldehyde transcardially. The brains were post-fixed with 4% formaldehyde overnight at 4 ^{o}C, rinsed in phosphate buffer, and vibratome sectioned sagittally at 200 μm. Sections were mounted on slides and X-gal stained. Tissue sections were photographed using an Olympus SZX12 or Zeiss Discovery V12 microscope.

For immunofluorescence, tissues were fixed as above except for the late gestation embryos or neonates, which were ex-sanguinated with cold PBS before brains were removed and immersion fixed. The fixed tissues were rinsed in PBS, cryoprotected in 30% sucrose overnight at 4 ^{o}C, embedded frozen in OCT and cryosectioned at 30-50 μm. For immunofluorescence staining, free floating sections or sections mounted on slides were incubated in the appropriate dilution of primary antibody in PBS/3% donkey serum/0.2% NP-40, followed by appropriate secondary antibody conjugated with Alexa Fluor 488, 594, or 647 (Molecular Probes). Mouse monoclonal antibodies used were: GFAP (1:400, Sigma-Aldrich, G3893), NeuN (1:1000, Chemicon, MAB377), PDGFRα (1:200, BD Biosciences), Lhx1/5 (1:100, Developmental Studies Hybridoma Bank, 4F2), and APC (1:100, Oncogene Sciences, clone CC-1). Rabbit polyclonal antibodies used were: GFP (1:500, Molecular Probes, A6455), Sox2 (1:3000, Chemicon), Calbindin (1:1000, Swant), TH (1:2500, Chemicon), Atoh1 (1:100, (Helms and Johnson, 1998)), Lhx2/9 (1:8000, a gift from T. Jessell), Pax2 (1:500, Zymed), and Olig2 (1:2000, Chemicon). Chick GFP (1:500, Aves lab), goat β-gal (1:500, Biogenesis), and guinea pig Sox10 (1:2000, gift M. Wegner) were also used. Rabbit anti-Ptf1a (1:10,000), guinea pig anti-Brn3a (1:10,000) and anti-Ascl1 antibody (1:10,000) were generated for this study using bacterially produced recombinant GST-fusion proteins as antigens (GST-Ptf1a plasmid provided by H. Edlund, Umea University, Sweden, and GST-Brn3a from E. Turner, Univ. of California, San Diego). Fluorescence imaging was carried out on a BioRad MRC 1024 confocal microscope. For each experiment multiple sections from at least 3 different animals were analyzed.

Neuroanatomical analyses

Nomenclature of anatomical structures for the adult brain was assigned using *The Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin, 2001) as a reference guide. For analysis of the embryonic brain, *The Atlas of Mouse*

Development (Kaufman, 1992), *Atlas of Prenatal Rat Brain Development* (Altman and Bayer, 1995), and *Chemoarchitectonic Atlas of the Developing Mouse Brain* (Jacobowitz and Abbott, 1997) were used as guides.

RESULTS AND DISCUSSION

Ascl1 lineage cells identify discrete populations of neurons as well as oligodendrocytes throughout the brain

In order to trace the fate of the transiently expressing Ascl1 progenitor cells into the mature brain, I used two BAC (bacterial artificial chromosome) transgenic mouse strains that have been previously described (Helms et al., 2005; Battiste et al., 2007) (Fig. 2.1). One strain, *Ascl1-GIC*, expresses constitutively active Cre recombinase in an Ascl1 pattern (Helms et al., 2005). I crossed *Ascl1-GIC* with *R26R-stop-lacZ* or *R26R-stop-YFP* Cre reporter mice to permanently label Ascl1 lineages, since Cre recombinase will excise the stop sequence upstream of LacZ or YFP (Soriano, 1999; Srinivas et al., 2001). Thus, at any given stage, β-gal or YFP positive cells are a cumulative representation of Ascl1 lineages up to that stage. In contrast, the second transgenic strain, *Ascl1-CreER^{TM}*, expresses an inducible Cre recombinase in the Ascl1 pattern, providing temporal control in labeling the Ascl1 lineages. Cre recombination is detectable within 6 hours following tamoxifen treatment, and it persists for approximately 24 h (Hayashi and McMahon, 2002), thus, only the progenitor cells expressing Ascl1 in a restricted time window will be labeled. Together, these mouse strains, combined with neuroanatomical analyses and immunofluorescence with cell-type specific markers, have allowed us to characterize the fate of Ascl1-expressing cells as they progress through development and settle in the mature brain (Fig. 2.1).

An *Ascl1-GIC;R26R-stop-lacZ* mouse brain was harvested at postnatal day 30 (P30). An X-gal stained parasagittal section illustrates that Ascl1 lineage cells make extensive but specific contributions to each major subregion in the brain (Fig. 2.2A). Higher magnification images reveal this specificity (Fig. 2.2B-H). X-gal stained cells were detected primarily in the granule cell layer in the olfactory bulb (Fig. 2.2B), in cells dispersed throughout the cortex (Fig. 2.2C), in the

striatum, septum and corpus callosum (Fig. 2.2D), and in presumptive noradrenergic cells in the locus coerulus (Fig. 2.2G). These results are consistent with previous studies on Ascl1 function in these lineages (Hirsch et al., 1998; Casarosa et al., 1999; Horton et al., 1999; Marin et al., 2000; Kim et al., 2007; Long et al., 2007). Furthermore, there were X-gal stained cells enriched in other brain regions including the preoptic area of the hypothalamus (Fig. 2.2E), the superior colliculus in the midbrain (Fig. 2.2F), and Purkinje cells in the cerebellum (Fig. 2.2H). Many X-gal stained cells were also found in white matter tracts, regions containing a high percentage of oligodendrocytes (Fig. 2.2D,H). The Ascl1 lineage cells were identified as neurons and oligodendrocytes since they co-label with the neuronal marker NeuN (Fig. 2.2I) and the oligodendrocyte marker Olig2 but not the astrocyte marker GFAP (Fig. 2.2J). This latter finding is consistent with the previous studies defining Ascl1 lineages in neurons and oligodendrocytes of the spinal cord and adult brain (Battiste et al., 2007; Kim et al., 2007; Parras et al., 2007).

Neurogenesis and gliogenesis are temporally dynamic. To reveal the fate of Ascl1 expressing cells from different stages of embryonic development, I utilized the tamoxifen-inducible Cre line, *Ascl1-CreER^{TM}*. *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos received tamoxifen by administering the drug to pregnant mice twice with a 6 hour interval on a given embryonic day. The CreER^{TM} recombinase is transiently activated and as the tamoxifen is cleared, the CreER^{TM} returns to the cytoplasm within 24-48 hours (Hayashi and McMahon, 2002). Embryonic or neonatal brains were harvested 7-8 days after tamoxifen administration, sectioned, and X-gal stained. An overview of Ascl1 lineage cells labeled as a consequence of tamoxifen administration at E10.5, E12.5, E15.5, and E17.5 demonstrate these cells distribute with distinctive patterns throughout the brain depending on the embryonic stage labeled (Fig. 2.3).

Tamoxifen administration at E10.5 resulted in X-gal positive cells that populate ventral regions of the forebrain such as the preoptic area (POA) in the hypothalamus, and amygdala (Fig. 2.3A and inset). The brain regions that had the highest contribution of Ascl1 lineage cells from this stage include the superior colliculus of the dorsal midbrain, and subdomains within the brainstem (Fig. 2.3A). With administration of tamoxifen 48 hours later at E12.5, the Ascl1 lineage shifts and populates additional forebrain regions including the olfactory bulb, striatum, and hippocampus (Fig. 2.3B). The superior colliculus was still strongly labeled, but in the hindbrain a strong contribution of cells was now found in the cerebellum. With tamoxifen at E15.5, Ascl1 lineage cells have dramatically decreased in brainstem regions but they continue to contribute to the olfactory bulb, striatum, POA, and hypothalamus in the forebrain (Fig. 2.3C). Also at this time, many scattered X-gal stained cells were detected in the cerebral cortex. These cells are likely inhibitory interneurons, consistent with the known birthdates of these cells in the ventral telencephalon migrating to the cortex (Miller, 1985; Miller and Nowakowski, 1988). Notably, oligodendrocytes begin to appear as illustrated by the dispersed X-gal stained cells found throughout the brain and the intensive staining in the corpus callosum (Fig. 2.3C). And finally by E17.5 tamoxifen administration, the Ascl1 lineage has largely shifted to oligodendrocytes rather than neurons. The identity of the dispersed cells as oligodendrocytes was confirmed by immunofluorescence staining with the marker Olig2 or Sox10 (data not shown). Continued Ascl1 lineage contribution to olfactory bulb neurons at this stage is a notable exception (Fig. 2.3D). A detailed examination of Ascl1 lineage cells contribution to major brain regions is provided in the following sections. In evaluating the following data, it is important to note that this in vivo genetic fate mapping paradigm is not 100% efficient and likely depends on the level of Cre expression and efficiency of recombination of the

Cre-reporter alleles. Because of this limitation, I believe I am preferentially marking the lineage of the highest Ascl1 expressing cells.

Ascl1 lineage in the telencephalon: The telencephalon gives rise to diverse brain structures such as the cortex and basal ganglia that contain diverse cell types including pyramidal neurons, interneurons and glia (Corbin et al., 2001; Marin and Rubenstein, 2001; Molyneaux et al., 2007). Neural bHLH transcription factors Neurog1 and Neurog2 (previously Ngn1 and Ngn2) and Ascl1 are expressed in the telencephalic germinal zones and are important in determining distinct neuronal cell types: glutamatergic (Neurog1 and Neurog2) versus GABAergic (Ascl1) (Fode et al., 2000). Briefly, Neurog2 expressing progenitors in dorsal telencephalon give rise to glutamatergic pyramidal neurons in the cerebral cortex via radial migration (Schuurmans et al., 2004). In contrast, Ascl1 expressing progenitors originate in the ganglionic eminences in the ventral telencephalon (Casarosa et al., 1999; Horton et al., 1999). Some of these progenitors migrate tangentially to generate GABAergic interneurons in the neocortex or the olfactory bulb (Fode et al., 2000; Parras et al., 2004; Long et al., 2007). Other Ascl1 progenitors migrate to contribute to interneurons in the striatum (Marin et al., 2000). Among the three major proliferative zones expressing Ascl1 in the ventral telencephalon: the medial (MGE), lateral (LGE) and caudal (CGE) (Fig. 2.4B',C') (Casarosa et al., 1999; Horton et al., 1999), X-gal staining in *Ascl1-CreER^{TM}* embryos predominantly marks LGE or CGE and not the MGE (Fig. 2.4B-C). Therefore, the Ascl1 lineages mapped here largely represent the cells originally from the LGE or CGE.

To map the Ascl1 lineages in the telencephalon at different stages of embryonic development, tamoxifen was administered to pregnant females at day 10.5 post coitum (10.5 dpc), 12.5 dpc or 15.5 dpc and *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos were harvested at E18.5. At each stage, Ascl1 lineage cells

contribute to neurons in the olfactory bulb consistent with the LGE origin of these cells (Marin and Rubenstein, 2001). There is a temporal shift with Ascl1 lineage cells contributing to the glomerular cell layers more intensely than the core granule cell layers when tamoxifen was administered at E10.5 or E12.5 (Fig. 2.4D-D'). In contrast, after E15.5, the Ascl1 progenitors are fated to the granule cell layers (Fig. 2.3C and 2.4D''). The generation of olfactory bulb interneurons from the Ascl1 lineage continues at E17.5 (Fig. 2.3D) and postnatally into the adult brain (Parras et al., 2004; Kim et al., 2007). In addition to the main olfactory bulb, the Ascl1 lineage also contributes to the accessory olfactory bulb (AOB) mainly from progenitors expressing Ascl1 prior to E12.5 (Fig. 2.4D).

For the ventral telencephalon, E10.5 and E12.5 Ascl1 lineage cells contribute to the striatum, piriform cortex and amygloid nucleus (Fig. 2.4E-G). Two days later, E12.5 Ascl1 lineage cells continue to contribute to these regions but now also include the septum (Fig. 2.4E'-G'). It is known that cortical interneurons arise from the ganglion eminences and migrate tangentially into the cortex (Corbin et al., 2001; Marin and Rubenstein, 2001). Starting after E12.5 tamoxifen administration, I begin to detect X-gal labeled cells in the cortex consistent with the appearance of these interneurons (Fig. 2.4I). The Ascl1 lineages also contribute to hippocampal neurons after E12.5 and continue through E15.5 (Fig. 2.4F'-G''). Furthermore, as predicted from the Ascl1 expression pattern and Ascl1 mutant phenotypes, Ascl1 lineage cells did not obviously contribute to glutamatergic projection neurons in the cortex (Fode et al., 2000).

A dramatic shift in cell-types derived from Ascl1 cells occurs after E15.5. At this later embryonic stage, Ascl1 progenitors largely become oligodendrocytes and populate white matter tracts such as the corpus callosum (Fig. 2.4J) as well as being scattered throughout the gray matter (Fig. 2.4E''-G''). There is a high density of Ascl1 lineage cells surrounding and emanating from the SVZ of the

lateral ventricles. Ascl1 continues to be expressed in the SVZ and in oligodendrocyte progenitors in the adult brain (Kim et al., 2007).

Ascl1 lineage in the diencephalon: Ascl1 expression is detected in distinct progenitor populations in the diencephalon, including the hypothalamus, prethalamus, thalamus, and pretectum (Fig. 2.4C-C') (Horton et al., 1999; Vue et al., 2007). Tamoxifen administration at E10.5 in *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos marks distinct progenitor zones in the diencephalon and allows us to follow the Ascl1 lineage in this region (Fig. 2.4C). Notably, most thalamic nuclei projecting to the cortex are not Ascl1 derived lineages, consistent with a recent report (Fig. 2.4F-G') (Vue et al., 2007). Instead, Ascl1 lineage cells are found in many other nuclei, such as the pretectal nuclei, reticular thalamic nucleus, a cluster of cells lateral to the habenular nucleus and substantia nigra (reticular part) (Fig. 2.4F-G'). The identity of the pretectal nuclei was verified by lack of Sox2 labeling (Vue et al., 2007; data not shown). Labeling at E15.5 no longer detects these nuclei; instead, scattered X-gal positive cells are found all over the diencephalon, representing oligodendrocytes (Fig. 2.4G'' and data not shown).

Ascl1 lineage cells in the dorsal midbrain show radial migration and sequentially become neurons in superior and inferior colliculi

The superior and inferior colliculi of the midbrain hold many histological parallels with the cerebral cortex. For example, both structures are multilayered, and neurogenesis contributes to each layer in an inside-out manner (Altman and Bayer, 1981a, 1981b; McConnell, 1995). However, neurogenic mechanisms in dorsal midbrain development are less well-studied compared to that in the cerebral cortex. Recent studies characterized the progenitor domains defined by patterns of transcription factors in the developing mesencephalon (Nakatani et al., 2007). Ascl1 expression is detected in all progenitor domains, suggesting Ascl1

derived progeny give rise to diverse neurons rather than a restricted neuronal subtype. Loss of Ascl1 function results in disruption of GABAergic neuron differentiation in this region (Miyoshi et al., 2004). However, the contribution of $Ascl1^{+}$ progenitors to other neuronal fates has not been investigated. Here I examined the developmental dynamics of Ascl1-defined progenitors for cellular lamination, migration and neuronal subtype.

Administration of tamoxifen at 11.5 dpc to pregnant female mice carrying *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos and analysis of embryos 24 hours later demonstrates the origin of these cells in the progenitor domains in the mesencephalon (Fig. 2.5A-A''). Analysis of tamoxifen-treated embryos 6-7 days later reveals some Ascl1 lineage cells migrate in radial arrays (Fig. 2.5B arrows, E) while others are more scattered and are consistent with tangential migration seen in the telencephalon (Fig. 2.5B arrowheads). To investigate the identity of the Ascl1 derived neurons, I used the POU homeodomain factor, Brn3a, recently shown to be a pan-glutamatergic neuronal marker (Nakatani et al., 2007). Whereas all marked cells are neurons (Fig. 2.5C), they distribute between $Brn3a^{+}$ and $Brn3a^{-}$ populations, suggesting $Ascl1^{+}$ progenitors not only give rise to GABAergic neurons as previously reported in dorsal midbrain (Miyoshi et al., 2004), but also to glutamatergic neurons (Fig. 2.5D). With these fate mapping studies, I cannot determine whether cells undergoing different migration patterns distribute between GABA and glutamatergic populations with specificity as has been demonstrated in the developing telencephalon. However, it has been reported that in the developing midbrain, the GABAergic neurons undergo a tangential migration while the glutamatergic neurons undergo a radial migration similar to the migratory pattern known for the cortex (Tan et al., 2002).

Within the mesencephalon, the superior and inferior colliculi comprise two main functional domains: mutisensory/visual and auditory processing, respectively (Dean et al., 1989). To determine how $Ascl1^{+}$ cells contribute to

these functional domains, I administered tamoxifen at 10.5, 12.5 or 15.5 dpc and analyzed embryos at E18.5. The superior colliculus contains cells derived from progenitor cells expressing Ascl1 during early stages of neurogenesis (~E10.5/E12.5) (Fig. 2.5F-G'', J). E10.5 Ascl1 lineage cells contribute to the intermediate layer, whereas E12.5 Ascl1 lineage cells populate both the deep and the superficial superior collicular neurons (Fig. 2.5F-F' arrows). In contrast, the inferior colliculus contains cells largely derived from progenitor cells expressing Ascl1 at late embryonic stages (E15.5) (Fig. 2.5H''). This temporal pattern was also illustrated in sagittal sections (Fig. 2.5J,K). The sequential generation of superior and inferior colliculi is consistent with early birthdating studies using ^{3}H-thymidine (Altman and Bayer, 1981a, 1981b). In addition, early born Ascl1 lineage cells also contribute to the mesencephalic trigeminal nucleus (Me5), the only primary sensory neuronal population in the CNS (Fig. 2.5F', arrowhead) (Louvi et al., 2007). In conclusion, Ascl1 lineage contribution to mesencephalon derived brain regions appears broader than other brain regions and encompasses multiple cell types with sequential contribution to rostral then caudal midbrain regions.

Ascl1 lineage cells in the cerebellum

The identification of specific neuronal subtypes that make up the cerebellum, their connections and their development has been extensively studied (Hatten et al., 1997; Wang and Zoghbi, 2001). Two bHLH transcription factors, Atoh1 (previously Math1) and Ptf1a, are major players in the generation of the glutamatergic granule neurons and GABAergic interneurons, respectively (Ben-Arie et al., 1997; Hoshino et al., 2005). Atoh1 is in the upper rhombic lip, a germinal epithelium of the dorsal interface around the fourth ventricle (Fig. 2.6B). Progenitor cells specified by Atoh1 migrate to the nuclear transitory zone (NTZ) where they express Lhx2/9 (Fig. 2.6C) and comprise the granule cell progenitors.

In contrast, Ptf1a is expressed in the ventricular zone of the cerebeller anlage (Fig. 2.6D). As these cells mature, they migrate to the cortical transitory zone (CTZ), express Lhx1/5, and contribute to all GABAergic neurons including Purkinje cells in the cerebellum (Glasgow et al., 2005; Hoshino et al., 2005; Chizhikov et al., 2006). Ascl1 is also in the ventricular zone of embryonic cerebellar anlage in an overlapping pattern with Ptf1a (Fig. 2.6B-D). At E12.5, Ascl1 and Ptf1a are in the ventricular zone where GABAergic neurons are generated, but are excluded from the rhombic lip where the glutamatergic granular cells originate from Atoh1 progenitors (Fig. 2.6B, D). Ascl1 positive cells do not express Lhx1/5, markers of the postmitotic neurons in the CTZ, suggesting Ascl1 expression is transient in this lineage similar to its expression characteristics in other regions (Fig. 2.6C).

To determine the fate of the Ascl1 lineage cells, tamoxifen was administered at different times during gestation to pregnant females carrying *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos. Analysis of *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos 24 hours after tamoxifen administration at E12.5 illustrates the origin of these cells in the ventricular zone (Fig. 2.6E). The fate of these progenitors at E17.5 was identified based on location and co-expression of the Purkinje cell marker, calbindin (Fig. 2.6F, H). Furthermore, when brains were harvested at P30, the X-gal stained cells had the distinct morphology of Purkinje cells (Fig. 2.6G-G').

Ascl1 expression in the ventricular zone of the cerebellar primodium starts as early as E10.5 and continues until E15.5 (Fig. 2.6B-C,J and data not shown). With tamoxifen administration earlier than E11.5 or later than E13.5, Ascl1 lineage cells preferentially became neurons in deep cerebellar nuclei (DCN) rather than Purkinje cells. *Ascl1-CreER^{TM};R26R-stop-lacZ* E17.5 brains exposed to tamoxifen at E14.5 revealed Ascl1 lineage cells contributing extensively to all three nuclei (medial, interposed, and lateral) (Fig. 2.6L) and were identified as neurons using co-labeling with NeuN, a pan-neuronal marker (Fig. 2.6M). DCN

contain both glutamatergic and GABAergic neurons. The Ascl1 lineage is restricted to a GABAergic fate determined by expression of Pax2, a GABAergic marker, but not the glutamatergic marker Tbr1 (Maricich and Herrup, 1999; Fink et al., 2006) (Fig. 2.6N and data not shown). This is consistent with Ascl1 expression restricted to the VZ, whereas the glutamatergic DCN neurons are derived from the rhombic lip cells (Fig. 2.6B) (Machold and Fishell, 2005; Fink et al., 2006).

Just as in other brain regions, Ascl1 expression in the cerebellum continues through late embryonic and prenatal stages. However, at these late stages, its expression is no longer restricted to the ventricular zone, but rather is detected throughout the cerebellum (Fig. 2.6P). A subset of these cells co-express the oligodendrocyte lineage marker Olig2, suggesting this population comprises glia progenitors rather than neuronal progenitors (Fig. 2.6P-P'). Exposure of E17.5 *Ascl1-CreER^{TM};R26R-stop-YFP* embryos to tamoxifen resulted in Ascl1 lineage cells mainly localized to white matter by P4 (Fig. 2.6Q). Few YFP expressing cells were located in the Purkinje cell layer or in deep cerebellar nuclei. The identity of these cells as oligodendrocytes was confirmed by co-labeling with Sox10 or PDGFRα (Fig. 2.6M-N). No overlap was detected with the astrocyte marker GFAP or BLBP, confirming Ascl1 expressing progenitors in the cerebellum do not give rise to astrocytes (data not shown).

Thus, Ascl1 is transient in progenitors to multiple discrete lineages in the cerebellum with a defined temporal sequence that follows the birthdate of these cells (Altman and Bayer, 1985a, 1985b, 1985c; Leto et al., 2006). In contrast to the midbrain, the Ascl1 lineage neurons in the cerebellum appear to be restricted to a GABA neurotransmitter phenotype and include Purkinje cells and DCN neurons. Co-expression of Ascl1 and Ptf1a in the cerebellum ventricular zone is consistent with this restriction to the GABAergic fate since Ptf1a is required for GABAergic neurons in both cerebellum and dorsal spinal cord (Glasgow et al.,

2005; Hoshino et al., 2005). As with other brain regions, at late embryonic stages, Ascl1 progenitors no longer give rise to neurons but rather to oligodendrocytes (Fig. 6Q-S and data not shown).

Ascl1 lineages give rise to the trigeminal brainstem nuclei

The lower rhombic lip in the hindbrain is a germinal zone located between the dorsal midbrain and the spinal cord. Progenitor cells from this region migrate extensively to contribute to distinct brainstem nuclei (Rodriguez and Dymecki, 2000). Recent studies have defined rhombic lip progenitor domains by expression of transcription factors patterned along the dorsoventral axis (Fig. 2.7J) (Landsberg et al., 2005; Sieber et al., 2007; Yamada et al., 2007). For example, it has been shown that progenitors expressing the bHLH transcription factor Atoh1 (dA1) are fated to populate nuclei sending mossy fibers that connect to cerebellar granule cells (Landsberg et al., 2005). Progenitors expressing Ptf1a (dA4) are fated to populate nuclei with climbing fibers that connect to Purkinje cells (Yamada et al., 2007). And finally, the homeodomain factor Lbx1 marks progenitors that contribute to somatosensory and viscerosensory relay neurons (dB1, dB3, dB4) in the brainstem (Sieber et al., 2007).

Ascl1 is present broadly from dA3 to dB3 throughout rhombomeres 2-7 (Fig. 2.7E) (Sieber et al., 2007). Similar to neurogenesis in spinal cord, Ascl1 progenitors are generated in two distinct neurogenic phases; early in a stripe-like pattern (E10/11) or late in a salt-and-pepper like pattern (E12/13) (Sieber et al., 2007). Analysis of *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos harvested 24 hours after E12.5 tamoxifen induction shows rapid differentiation and migration of Ascl1 progenitors to the mantle zone, compared to the restricted expression of Ascl1 in the ventricular zone (Fig. 2.7B-C). Although Ascl1 is present broadly in the VZ of the lower rhombic lip, in *Ascl1-CreER^{TM}* embryos, CreER^{TM} is restricted to the dB3 population prior to E12.5 (Fig. 2.7E and K). This is evident

by YFP from *Ascl1-CreER^{TM};R26R-stop-YFP* overlap with Tlx3 and Lmx1b, but not Pax2 and Lhx1/5 after tamoxifen administration E10.5 and analysis of E11.5 embryos (Fig. 2.7F-I). Furthermore, after E12.5, the dBLb subset of the Ascl1 lineage is labeled (Fig. 2.7K' and data not shown). Detection of only a subset of the Ascl1 lineage in the brainstem in this transgenic strain is consistent with results seen in the spinal cord where dI3 and dI5 population but not dI4 were preferentially marked (Battiste et al., 2007). This may reflect differences in level of expression of Ascl1 in these lineages. Nevertheless, the Ascl1 lineages mapped here represent the fate of progenitors from dB3 and dBLb.

Ascl1-CreER^{TM};R26R-stop-lacZ embryos were exposed to tamoxifen at either E10.5 or E12.5, and the Ascl1 lineages in brainstem nuclei were examined at E17.5/E18.5. Ascl1 lineage cells contribute to the trigeminal sensory nuclear complex ranging from the pons into the caudal medulla (Qian et al., 2002). Ascl1 dB3 and dBLb progenitors populate the spinal trigeminal nucleus (Sp5) consistent with previous studies of Lbx1 (Fig. 2.7L-N') (Sieber et al., 2007). In addition, Ascl1 lineage cells include the primary sensory nucleus (Pr5), and other associated nuclei such as the parvicellular reticular nucleus (PCRtA), and the gigantocellular nucleus (Gi) (Fig. 2.7L-N'). Notably, since the mesencephalic trigeminal nucleus (Me5) in dorsal midbrain is also derived from the Ascl1 lineage, Ascl1 progenitors appear to preferentially contribute a functional network for the somatosensory relay system (Fig. 2.5F'). In contrast, Ascl1 progenitors in dB3 and dBLb are not fated to the six precerebellar nuclei (pontine gray, reticulotegmental, vestibular, lateral reticular, external cuneate, and inferior olivary nuclei) (Fig. 2.7L-N''). Thus, *Ascl1-CreER^{TM}* transgenic mice have allowed us to determine the fate of a subpopulation of the Ascl1 progenitors in the developing brainstem.

Concluding Remarks

In this study, I describe the temporal fate map for Ascl1 lineages throughout the brain. A summary of the CNS structures that receive contribution from Ascl1 lineage cells is provided in Table 2.1. Neuronal progenitors marked by CreER^{TM} in *Ascl1-CreER^{TM}* are dynamic in that they are transitioning from proliferating progenitors to differentiating neurons. This is inferred from experiments where embryos were harvested within 24 hours of induction by tamoxifen and lineage marked cells were already found lateral to progenitor domains and express markers of differentiating markers. This is similar to what was seen in embryonic spinal cord and adult neurogenesis (Battiste et al., 2007; Kim et al., 2007), and is consistent with the interpretation that cells with high Ascl1 levels, as would be preferentially labeled in this paradigm, are differentiating. Indeed, overexpression of Ascl1 in chick neural tube induces progenitors to rapidly exit the cell cycle, move out of the VZ and express markers of neuronal differentiation (Nakada et al., 2004).

Ascl1 progenitors give rise to diverse neuronal subtypes including GABAergic (Horton et al., 1999; Fode et al., 2000), glutamatergic (Helms et al., 2005), serotonergic (Pattyn et al., 2004), noradrenergic (Hirsch et al., 1998) and acetylcholinergic (Marin et al., 2000). This is in contrast to other transcription factor marked populations such as Atoh1 and Tlx3 which appear restricted to glutamatergic neurons, and Pax2 which is restricted to GABAergic lineages. Ascl1 does appear to function in neuronal specification but this is region dependent. For example, in early dorsal spinal cord, Ascl1 is required for dI3 and dI5 and overexpression results in excess dI3 and dI5 (Helms et al., 2005). In the second round of neurogenesis in the dorsal spinal cord, Ascl1 is important for generating normal numbers of the GABAergic dIL^{A} neurons (Mizuguchi et al., 2006; Wildner et al., 2006). Identifying interacting factors that work with Ascl1 will be important for understanding how it functions to generate neuronal diversity.

Ascl1 is present in progenitors to oligodendrocytes in the embryonic spinal cord, and in embryonic and adult brain. However, not all oligodendrocytes arise from Ascl1 expressing cells since the earliest oligodendrocytes arise in the ventral neural tube from non-Ascl1 cells (Lu et al., 2002; Zhou and Anderson, 2002; Sugimori et al., 2008). However, in each brain region examined, the late stage expression of Ascl1 is preferentially marking oligodendrocyte progenitors, not neuronal progenitors or astrocytes. The function of Ascl1 in oligodendrocyte development is just beginning to be uncovered. Oligodendrocytes in Ascl1 mutant mice fail to express the full complement of known markers such as Nkx2.2 (Sugimori et al., 2008).

To interpret in vivo lineage studies using inducible Cre recombinase in transgenic mice, it is important to understand the limitations of this paradigm. First, there is always the caveat that expression from the transgene is not 100% reliable due to position effects or lack of regulatory information. The BAC used to generate the transgenic mice includes over 100 kb of sequence both 5' and 3' flanking the *Ascl1* coding region, increasing the likelihood that important transcription control regions are included. However, I know some Ascl1 lineages are not detected such as cells derived from the MGE in the telencephalon, and a subset of neurons in the spinal cord and brainstem. Second, the induction of Cre is not expected to be 100% efficient in deleting the STOP sequence from the reporter so it is not expected that 100% of the cells derived from Ascl1 will be marked. However, in a majority of cases, expression from this BAC transgenic line appears to reflect endogenous Ascl1 expression, and thus, identifying X-gal or YFP marked cells in a specific brain region provides strong support that these cells are derived from Ascl1-expressing progenitor cells.

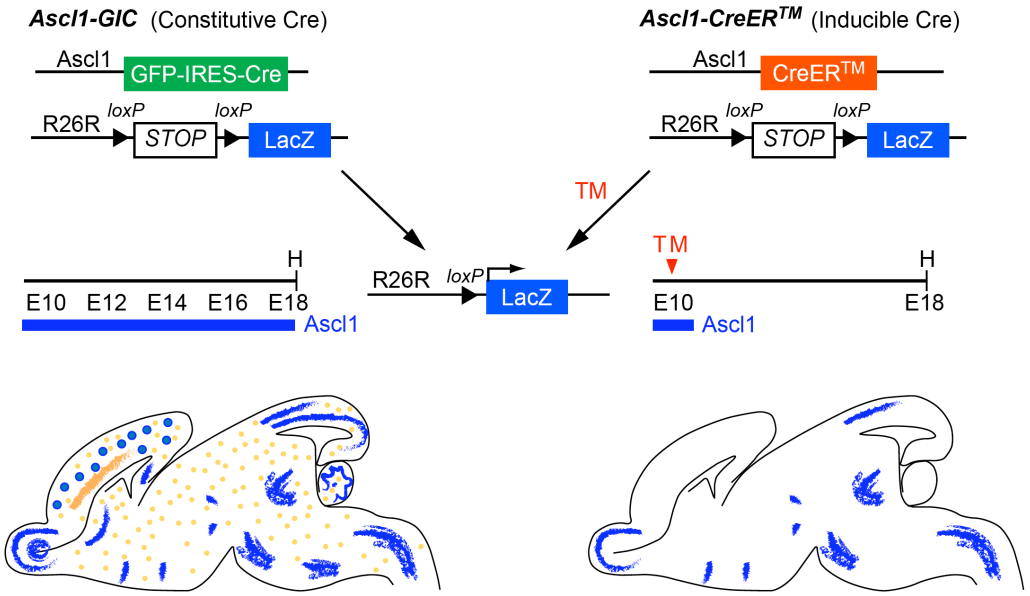

Figure 2.1. Diagram of Ascl1 transgenic mice and the fate-mapping strategy. Two transgenic mouse models were generated with a BAC containing Ascl1 >200 kb flanking non-coding sequence. *Ascl1-GIC* replaces the Ascl1 coding sequence with GFP-IRES-Cre (Helms et al., 2005). This strain will reveal an accumulation of Ascl1 lineage cells when crossed with a Cre reporter mouse strain such as *R26R-stop-lacZ* (Soriano, 1999). *Ascl1-CreER*^{TM} replaces the Ascl1 coding sequence with an inducible Cre (Battiste et al., 2007). Only Ascl1 lineage cells originating at the time of tamoxifen (TM) treatment will be detected when crossed with *R26R-stop-lacZ*. Sagittal views of mouse brains from each paradigm are diagrammed to highlight that the inducible Cre will reveal only a subset of the Ascl1 lineage. Blue represents neurons and orange represents oligodendrocytes E, embryonic stage; H, harvest age.

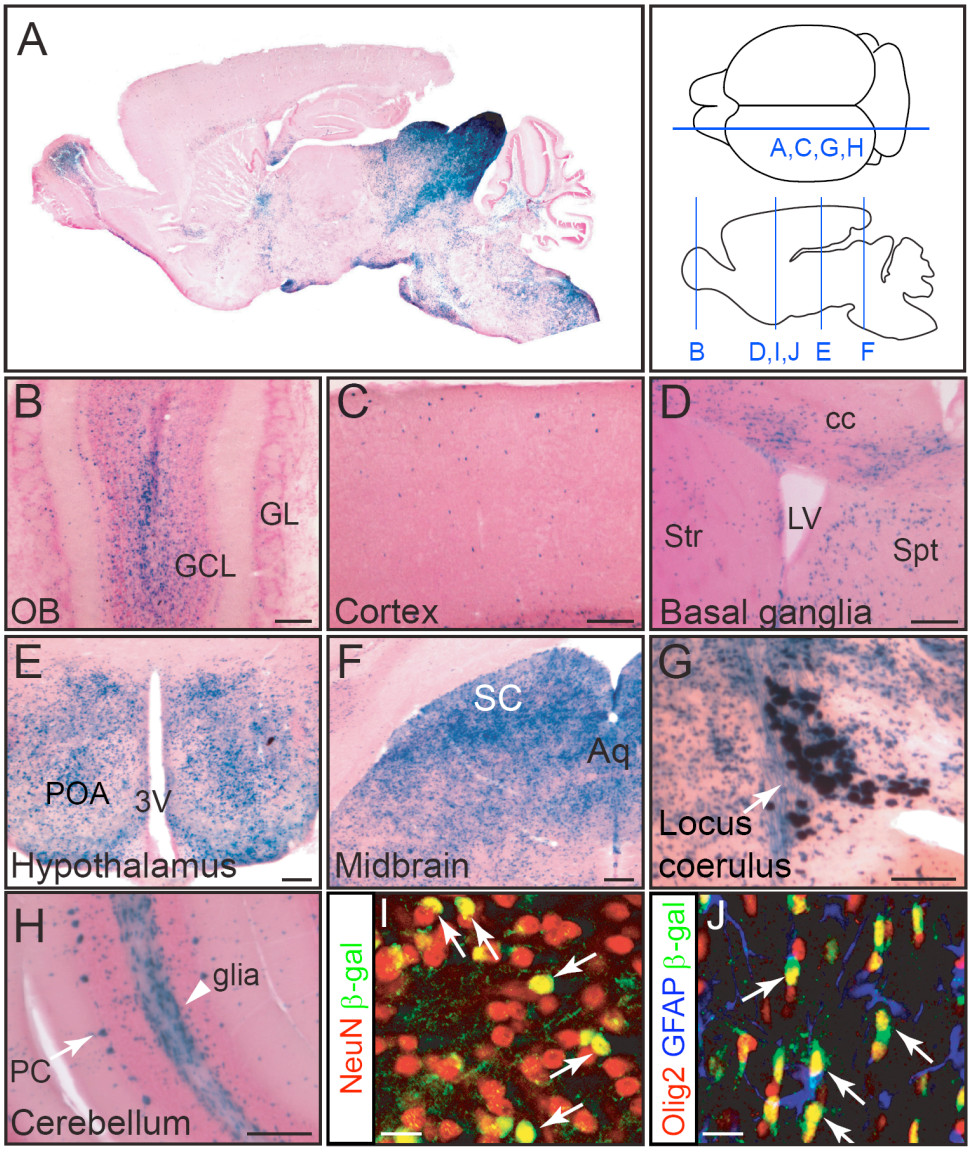

Figure 2.2. Ascl1-expressing cells give rise to discrete cell populations in brain. (A-H) X-gal staining of P30 brains from *Ascl1-GIC;R26R-stop-lacZ* transgenic mice including (A) whole brain, (B) olfactory bulb, (C) cerebral cortex, (D) striatum, (E) preoptic area of the hypothalamus, (F) dorsal midbrain, (G) locus coerulus, and (H)

cerebellum. (I, J) Immunofluorescence for β-galactosidase in the striatum (I) or the anterior commissure (J). ß-gal cells co-express neuronal marker NeuN (I, arrows) or the oligodendrocyte marker Olig2 (J, arrows, but not astrocytes (GFAP). Diagram depicts sectioning plane for each panel. 3V, third ventricle; Aq, aquaduct; cc, corpus callosum; GCL, granule cell layer; GL, glomerular layer; LV, lateral ventricle; OB, olfactory bulb; PC, Purkinje cells; POA, preoptic area; SC, superior colliculus; Spt, septum; Str, striatum. Scale bars = 200μm.

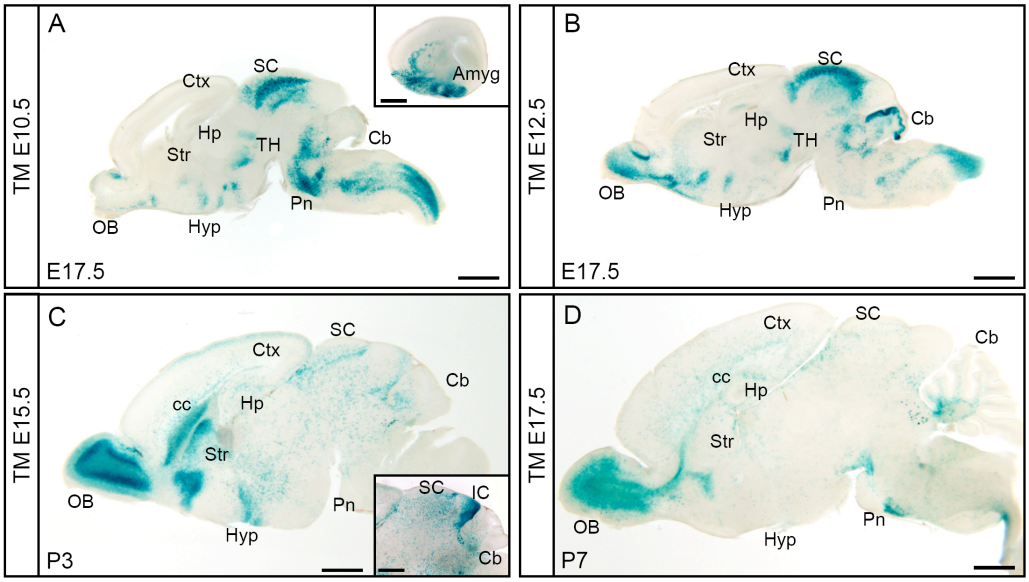

Figure 2.3. Temporal specific fate maps of the Ascl1 lineage in brain.
Sagittal views of X-gal staining of *Ascl1-CreER^{TM};R26R-stop-lacZ* brains at indicated stages treated with tamoxifen (TM) at embryonic stages indicated. Insets in A and C show amygdala and superior/inferior colliculus from different parasagittal axis sections respectively. Amyg, amygdala; Cb, cerebellum; cc, corpus callosum; Ctx, cortex; Hp, hippocampus; Hyp, hypothalamus; IC, inferior colliculus; OB, olfactory bulb; Pn, Pons; SC, superior colliculus; Str, striatum; TH, thalamus. Scale bars= 1000µm.

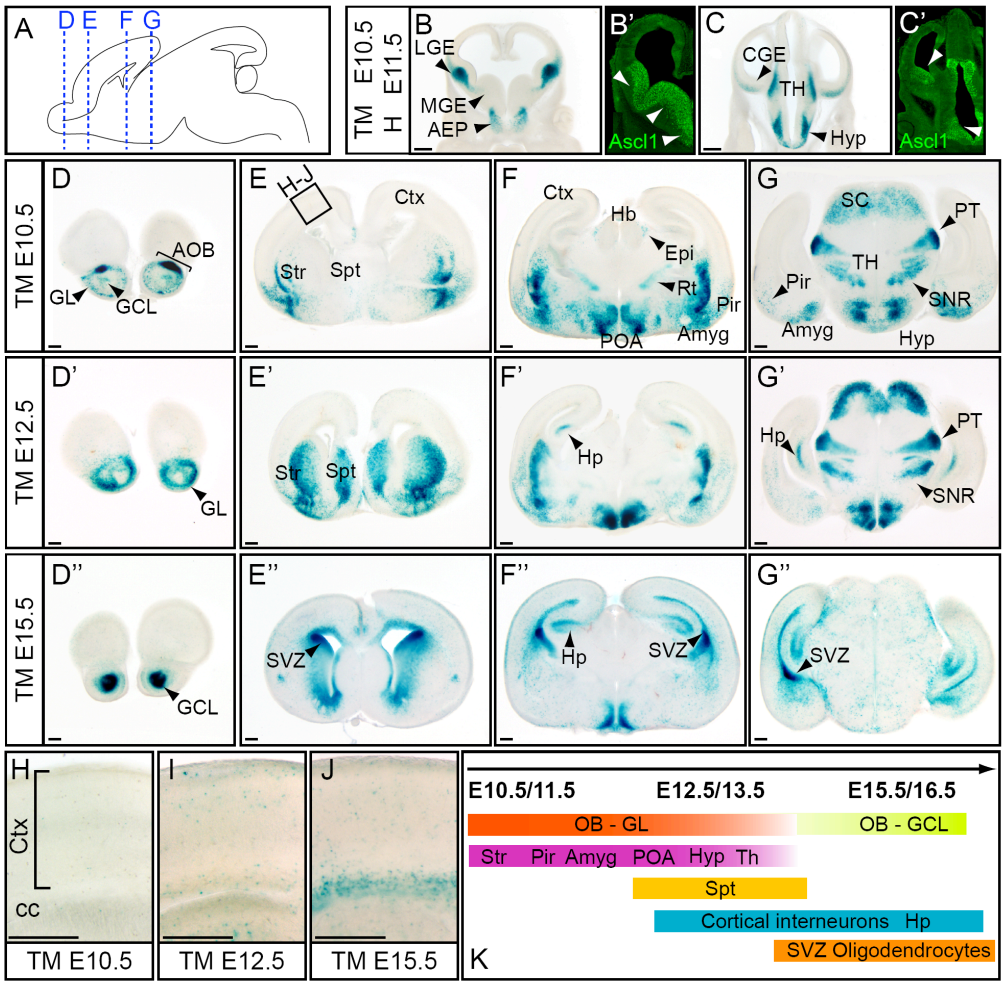

Figure 2.4. Ascl1 lineage in the forebrain.

(A) Schematic of an E18.5 sagittal brain where dotted lines indicate coronal planes used for panels D-G''. (B-C) X-gal stained coronal sections of telencephalon in *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos treated at E10.5 and harvested 24 hr later at E11.5 showing the cells of origin marked by *Ascl1-CreER^{TM}* compared to the endogenous expression patterns of Ascl1 (B',C'). *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos exposed to tamoxifen at E10.5 (D-G), E11.5 (D'-G'), and E15.5 (D''-G'') and harvested at E18.5. (D-D'') (H-J) Magnified views (see box in E) to show Ascl1 lineage cells in cortical interneurons and the corpus callosum after E12.5. (K) Summary of the temporal specific generation of discrete Ascl1 lineage cells in forebrain. AEP, anterior entopeduncular area; AOB, accessory olfactory bulb; Amyg, amygdala; cc, corpus callosum; CGE,

caudal ganglionic eminence; Ctx, cortex; Epi, epithalamus; GCL, granule cell layer; GL, glomerular layer; Hb, habernula; Hyp, hypothalamus; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Pir, piriform cortex; POA, preoptic area; PT, pretectal nucleus; Rt, reticular thalamic nucleus; Sc, superior colliculus; SNR, substantia nigra (reticular part); Spt, septum; Str, striatum; SVZ, subventricular zone; TH, thalamus. Scale bars = 300μm.

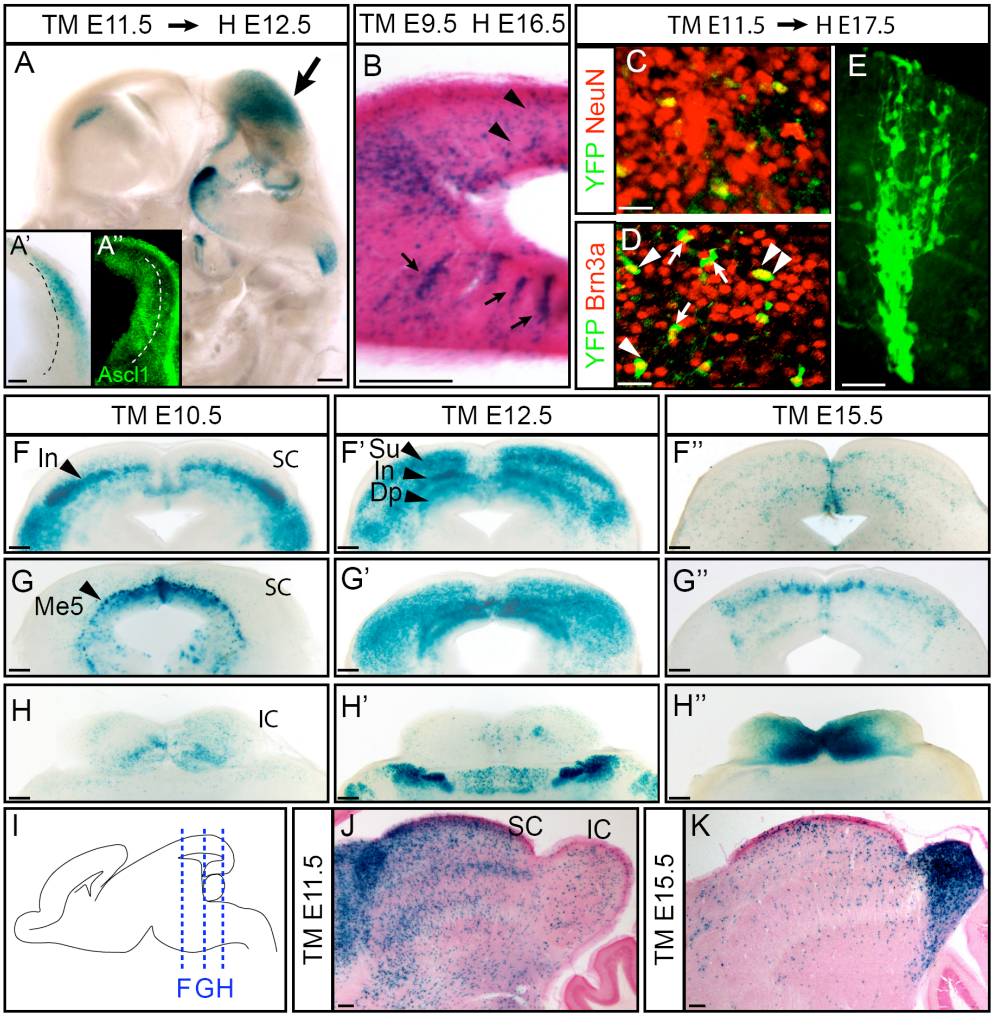

Figure 2.5. Ascl1 lineage in the dorsal midbrain.
(A) β-gal expression in dorsal mesencephalon (arrow) of *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos tamoxifen-induced (TM) at E11.5 and harvested (H) at E12.5. Higher magnification of a coronal view shows ß-gal present in cells adjacent to the ventricular zone (A'). In contrast, endogenous Ascl1 expression is restricted to the ventricular zone (A''). (B) Ascl1 lineage cells show both scattered (arrowheads) and radially (arrows) arrayed expression patterns. (C-E) In *Ascl1-CreER^{TM};R26R-stop-YFP* embryos, YFP^{+} Ascl1 lineage cells are neurons (NeuN^{+}) and some express the glutamatergic marker Brn3a (arrowheads indicate co-expressing cells, arrows indicate no co-expression). (F-H) X-gal stained coronal sections of superior and inferior colliculi from rostal to caudal

(from F to H). *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos were exposed to tamoxifen as indicated and harvested at E18.5. (I) Schematic view of sectioning plane for panels in (F-H''). (J-K) P30 brains of *Ascl1-CreER^{TM};R26R-stop-lacZ* showed sequential contribution of the Ascl1 lineage to superior and inferior colliculi from early versus late embryogenic stages. IC, inferior colliculus; Me5, mesencephalic trigeminal nucleus; SC, superior colliculus. Scale bars = 20μm for C-E, 200μm for A-B, F-K.

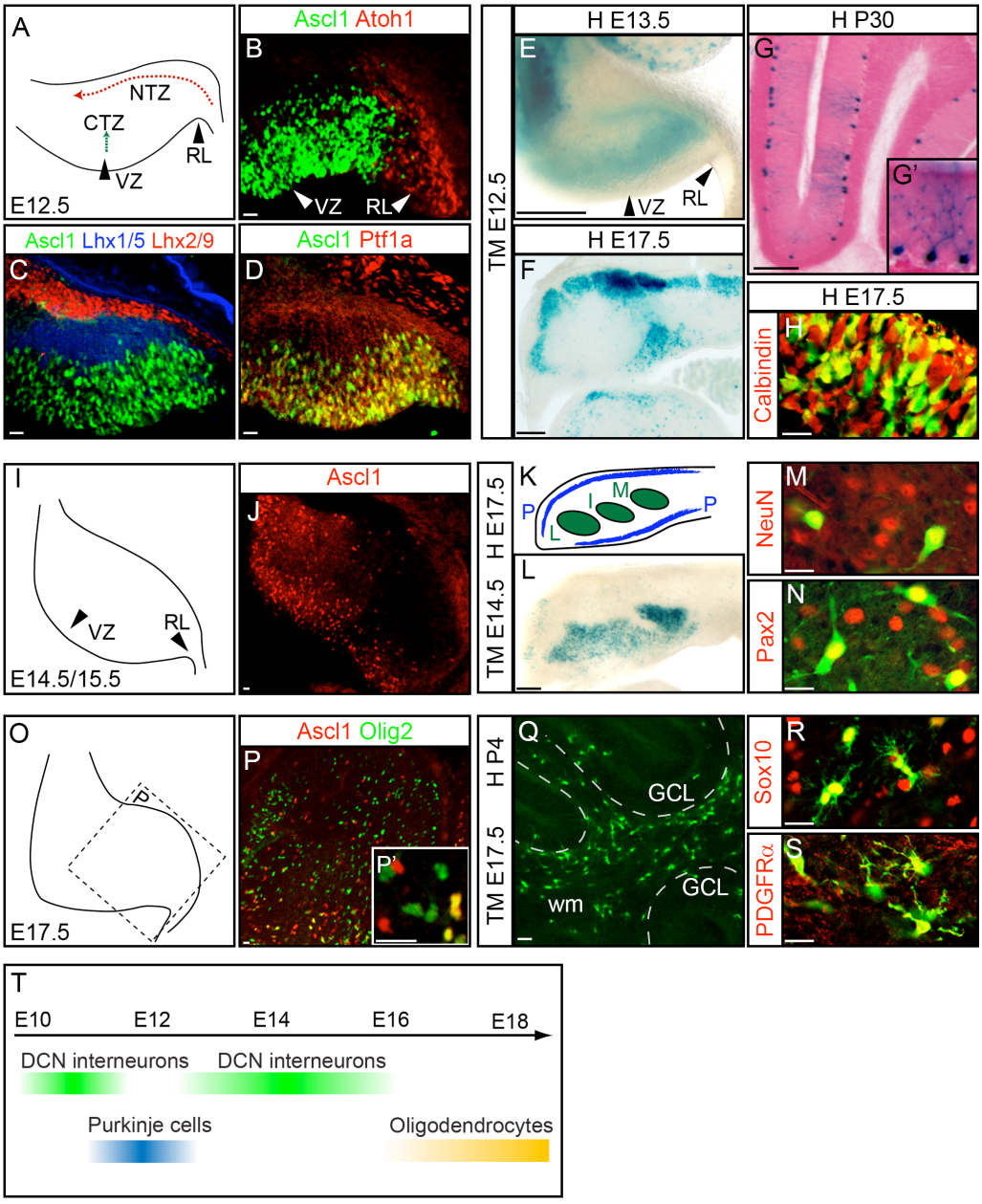

Figure 2.6. The Ascl1 lineage generates Purkinje cells, deep cerebellar nuclei interneurons and oligodendrocytes at specific stages in the cerebellum.
(A) Diagram depicting E12.5 cerebellum primodium for panels (B-D). Progenitors from the ventricular zone migrate to the cortical transitory zone (CTZ), whereas progenitors from the rhombic lip migrate to the nuclear transitory zone (NTZ). (B-D) Ascl1 is in the

ventricular zone (VZ) distinct from Atoh1 in rhombic lip (RL). (C) Transient expression of Ascl1 is not overlapped by CTZ marker Lhx1/5 or NTZ marker Lhx2/9. (D) Ascl1 and Ptf1a are co-expressed in VZ. (E-H) *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos exposed to tamoxifen at E12.5 and harvested at E13.5 (E), at E17.5 (F,H) or P30 (G). The identity of Purkinje cells are confirmed with a magnified view showing the morphology of Purkinje cells (G') at P30 or with co-expression of Purkinje cell marker Calbindin (H). (I) Diagram depicting E14.5/E15.5 cerebellum primodium. (J) Ascl1 expression in the VZ of the E15.5 cerebellum. (K) Coronal section of cerebellum with Purkinje cell layer (P) and three deep cerebellar nuclei (L,I,M) at E17.5. (L) β-gal^{+} Ascl1 lineage cells marked at E14.5 populate all three DCN nuclei, and co-label with neuronal marker NeuN (M) and interneuron marker Pax2 (N). (O) Diagram depicting E17.5 cerebellum primodium. (P) Scattered expression of Ascl1 progenitors in E17.5 cerebellum co-express oligodendrocyte marker Olig2 (P'). (Q-S) Ascl1 progenitors marked at E17.5 and detected at P4 in *Ascl1-CreER^{TM};R26R-stop-YFP* cerebellum shows most of the Ascl1 lineage in white matter (Q) and co-express Sox10 (R) and PDGFRα (S). (T) Summary diagram depicting temporal specific contribution of Ascl1 lineage cells in cerebellum. CTZ, cortical transitory zone; DCN, deep cerebellar nucleus; GCL, granule cell layer; I, interposed DCN; L, lateral DCN; M, medial DCN; NTZ, nuclear transitory zone; P, Purkinje cells; RL, rhombic lip; VZ, ventricular zone; wm, white matter. Scale bars = 20μm for B-D, H, J, M-S and 200μm for E-F, L.

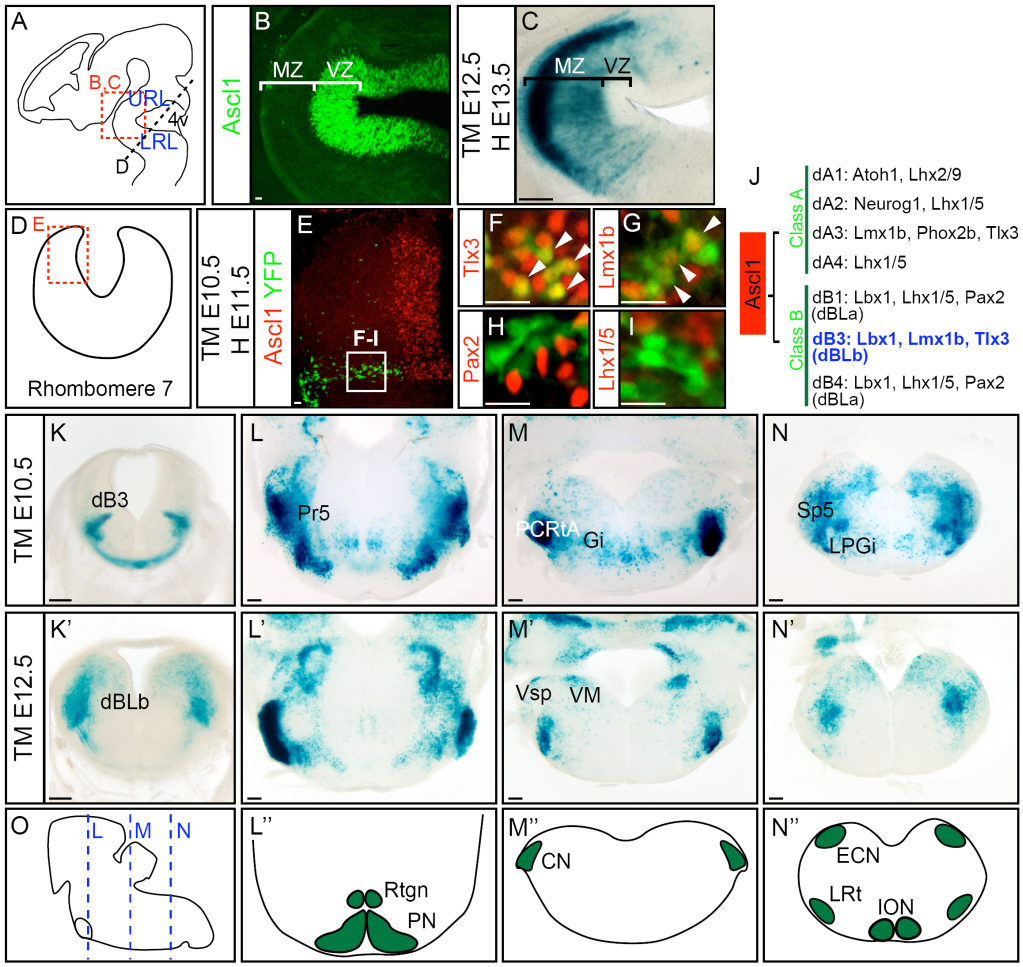

Figure 2.7. Ascl1 lineage in the trigeminal sensory system in the brainstem.
(A) Diagram of an E12.5 embryo depicting section planes in hindbrain regions shown in (B-D). (B) Ascl1 is restricted to the ventricular zone around the fourth ventricle whereas β-gal marked Ascl1 lineage cells from E12.5 in *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos are found in the mantle zone by E13.5. (E-I) YFP^{+} Ascl1 lineage cells in *Ascl1-CreER^{TM};R26R-stop-YFP* treated with tamoxifen at E10.5 and harvested at E11.5 are dB3 neurons as indicated by co-expression with Tlx3 (H) and Lmx1b (G), but not Pax2 (H) or Lhx1/5 (I). (J) Summary of neuronal subtypes and transcription factors for early (dA1-dB4) and late (dBLa and dBLb) neurogenesis in rhombomere 7 (Sieber et al., 2007). (K-N') In *Ascl1-CreER^{TM};R26R-stop-lacZ* embryos, tamoxifen was administered at E10.5 (K-N) and E12.5 (K'-N'), and harvested 24 hours later (K, K') or at E18.5 (L-N'). (O) Diagram depicting E18.5 embryo brainstem with section plane used in panels (L-N') shown. (L''-N'') Diagrams of coronal sections of hindbrain showing precerebellar nuclei.

4v, fourth ventricle; CN, cochlear nucleus; ECN, external cuneate nucleus; Gi, gigantocellular reticular nucleus; ION, inferior olive nucleus; LPGi, lateral paragigantocellular nucleus; LRL, lower rhombic lip; LRt, lateral reticular nucleus; MZ, mantle zone; Sp5, spinal trigeminal nucleus; PCRtA, parvicellular reticular nucleus, alpha part; PN, pontine nuclei; Pr5, the primary sensory trigeminal nucleus; Rtgn, reticulotegmental nucleus; URL, upper rhombic lip; VM, medial vestibular nucleus; Vsp, spinal vestibular nucleus; VZ, ventricular zone. Scale bars = 20μm for B, E-I, 200μm for C, K-N'.

Table 2.1. Neural derivatives of Ascl1 lineage in CNS

Neuron & Oligodendrocyte (Battiste 2007, Parras 2007, Sugimori 2007&2008)

	Anatomical Structure	Cell Types/Nuclei	References
Forebrain	Olfactory Bulb :(MOB and AOB)	Interneurons in granule cell and glomerular layer	This study, Long 2007, Parras 2004
	Cortex	cortical interneurons	This study, Casarosa 1999
	Ventral limbic system (Striatum, Amygdala)	Cholinergic or CR^+ Interneurons	This study, Marin 2000
	Hippocampus	Pyramidal and granule neurons	This study, Pleasure 2000
	Preoptic Area	ND	This study
Midbrain	Superior/Inferior Colliculus	Both glutamateric and GABAergic neurons	This study, Miyoshi 2004, Nakatani 2007
	Thalamus	Thalamic nuclei (Epi, Pre, Rt)	This study, Tuttle 2000, Vue 2007
	Hypothalamus	POMC neurons	Mcnay 2006
	Trigeminal sensory system	Me5	This study
Hindbrain	Cerebellum	deep cerebellar nuclei interneurons, Purkinje cells	This study
	Trigeminal sensory system	Spinal trigeminal nucleus (Sp5), associated nuclei (Pr, Gi, PCRtA)	This study
	Locus coeruleus	Noradrenergic neurons	This study, Hirsch 1998
	Dorsal raphe nucleus	Serotonergic neurons	Pattyn 2004
Spinal Cord		dI3,5, v2, $dIL_{A\&B}$ neurons	Helms 2005, Li 2005, Wildner 2006

* AOB, accessory olfactory bulb; CR, calrethnin; Epi, epithalamus; Gi, gigantocellular reticular nucleus; Me5, mesencephalic trigeminal nucleus; MOB, main olfactory bulb; PCRtA, parvicellular reticular nucleus; Pr, pontine reticular nucleus; Pre, pretectal thalamus; Rt, reticular thalamus.

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CHAPTER THREE

Distinct neuroglial lineages and functions of Ascl1 at different cell fate decision nodes in the developing spinal cord

INTRODUCTION

The neuronal network in the dorsal horn of the spinal cord is responsible for integrating and relaying somatosensory information from the peripheral sensory neurons to higher centers in the central nervous system (CNS). To generate its proper functional architecture, the correct numbers of excitatory and inhibitory neurons and glia must be generated in precise temporal and spatial manner. The ventricular zone of the dorsal neural tube generates all three neural cell types: neurons, oligodendrocytes, and astrocytes (Pringle et al., 1998; Helms and Johnson, 2003; Cai et al., 2005). From birthdating studies, it is known that these cell types arise at different times in embryogenesis (Kessaris et al., 2001). At an early phase of neurogenesis (E10-11.5), dorsal progenitors generate dI1-dI6 interneurons that mostly reside in the deep dorsal horn whereas a late phase of neurogenesis (E11.5-13) generates inhibitory dIL^A and excitatory dIL^B populations, the major neuronal subtypes in the superficial layers of the dorsal horn (reviewed by Caspary and Anderson, 2003; Helms and Johnson, 2003). After neurogenesis, gliogenesis predominates from the dorsal ventricular zone at late stages of embryogenesis (E14-E18) (Kessaris et al., 2001). The in vivo understanding of how these distinct cell types are generated from asymmetric and symmetric divisions, and the molecular basis controlling these lineage decisions, is lacking.

Ascl1 (previously Mash1), a neural basic helix-loop-helix (bHLH) transcription factor, is expressed in neural precursors transitioning from proliferating stem/progenitor stages to differentiating stages for specific cell types in both neurogenic and gliogenic periods (Battiste et al., 2007; Kim et al., 2008). The transient nature of Ascl1 expression at cell fate decision points proposes Ascl1 as a potent molecular probe to analyze the dynamics of lineage development. *Ascl1* is a mammalian homolog of the *Drosophila* proneural genes of Achaete-scute complex, players in the Notch signaling network, a signaling pathway known to regulate binary cell fate decisions during development (Johnson et al., 1990). Extensive analyses using *Ascl1* null mice or overexpression paradigms demonstrated its essential functions in the differentiation and specification of distinct neuronal or glia cell types in many regions throughout CNS and peripheral nervous system (PNS) (Horton et al., 1999; Perez et al., 1999; Fode et al., 2000; Parras et al., 2002; Nakada et al., 2004; Parras et al., 2004; Pattyn et al., 2004; Helms et al., 2005; Wildner et al., 2006; Battiste et al., 2007; Parras et al., 2007; Sugimori et al., 2008). In addition, identification of genes encoding Notch ligands such *Dll1* or *Dll3* as in vivo transcriptional targets of Ascl1 suggests that its interplay with the Notch signaling pathway is a major mechanism controlling diverse aspects of the vertebrate neural development (Castro et al., 2006; Henke et al., 2009).

Here I investigated the temporal description of the lineage relationship between neurogenesis and gliogenesis by fate mapping distinct Ascl1^{+} progenitor populations using the cre-flox recombination system. Ascl1 is present in the ventricular zone in neuronal and glial lineage restricted progenitors, but not in bipotential progenitors to these cell types in the neural tube. In neuronal lineage, Ascl1^{+} progenitors consist of at least two temporally defined populations. The earlier lineage Ascl1^{+} cell gives rise to both dIL^{A} and dIL^{B} neuronal populations, whereas cells expressing Ascl1 slightly later in the lineage are restricted to the

dIL^{B} neuronal fate. Likewise, Ascl1^{+} glial progenitors also consist of two temporally defined populations: Ascl1 in cells in a developmentally earlier state give rise to both astrocytes and oligodendrocytes, whereas Ascl1 in cells at a later phase are restricted to the oligodendrocyte lineage. Using a conditional *Ascl1* knockout paradigm, distinct cell autonomous functions of Ascl1 in each lineage were determined. There was a general defect in differentiation of progenitor cells to neurons or oligodendrocytes. In addition, conditional loss of *Ascl1* revealed the loss of cells in only one arm of each lineage at the different timepoints. During neurogenesis dIL^{A} but not dIL^{B} neurons were decreased, and during gliogenesis oligodendrocytes but not astrocytes were lost. These results demonstrate that Ascl1 is crucial for neuronal cell type diversification, and neural subtype specification, playing important roles at several different nodes of cell fate decisions throughout neurodevelopment.

MATERIALS AND METHODS

Generation of *Ascl1*^{CreERT2} knock-in mouse

Ascl1^{CreERT2} knock-in mice were generated by replacing the *Ascl1* coding region with *CreER*^{T2} (provided by P. Chambon) (Indra et al., 1999) and Frt-Neo-Frt cassettes. The targeting strategy was the same used to generate *Ascl1*^{GFP} knock-in mice (Leung et al., 2007). The long arm of the targeting vector included a 5.4 kb SacI genomic DNA fragment including the *Ascl1* translation start site. The endogenous ATG was replaced by a short sequence containing a PacI site and a consensus Kozak site. A 1.6 kb genomic DNA fragment from BamHI (in the 3' end of the coding sequence) to an SpeI site located downstream was used as the short arm. The CreER^{T2}-Frt-Neo-Frt cassette was inserted between the 5' and 3' arms in the pBC targeting vector containing the thymidine kinase (TK) gene driven by a Pgk promoter. As a by-product of cloning, 50 bp exogenous sequence was introduced in front of the Kozak sequence just as in the original *Ascl1*^{GFP} targeting vector.

The CreER^{T2} targeting vector was linearized by digestion with PmeI and introduced into embryonic stem cells. Following positive-negative selection with Neo and TK, the resistant colonies were screened by Southern blot analysis of EcoRI digested DNA using 5' and 3' probes. The correctly targeted clones were injected into C57BL/6 blastocysts, which were transferred to the uterus of pseudopregnant females. After obtaining germ line transmission from the resulting chimeric male mice, *Ascl1*$^{CreERT2\text{-}Frt\text{-}Neo\text{-}Frt}$ mice were crossed with FLPe mice (Rodriguez et al., 2000) to remove the neomycin cassette.

For PCR genotyping, the following primers were used: 5'-AAC TTT CCT CCG GGG CTC GTT TC-3' (Sense Ascl1 5'UTR) and 5'-CGC CTG GCG ATC CCT GAA CAT G-3' (Anti sense Cre) giving a PCR product of 247 bp.

Transgenic mice and Tamoxifen treatment

Ascl1-CreER^{TM}, *Ascl1^{GFP}* ,*Glast^{CreERT2}*, *Nestin-CreER^{T2}*, *R26R-stop-YFP(or LacZ)*, *Tau$^{mGFP-LacZ}$* mice were described previously. *Ascl1-CreER^{TM}* is a BAC transgenic mouse where CreER^{TM} replaces the *Ascl1* coding sequence (Battiste et al., 2007). *Ascl1^{GFP}* has GFP knocked into the *Ascl1* locus replacing the *Ascl1* coding sequence and otherwise only modifying the locus with a short linker sequence around the ATG (Leung et al., 2007). *Glast^{CreERT2}* is an inducible Cre knock-in strain where CreERT2 is inserted in the locus of GLAST, astrocyte-specific glutamate transporter (Mori et al., 2006). *Nestin-CreER^{T2}* is the transgenic mouse expressing CreERT2 under the control of the rat nestin promoter and enhancer (Battiste et al., 2007; Lagace et al., 2007). *R26R-stop-YFP and R26R-stop-LacZ* are Cre recombinase reporter strains (Srinivas et al., 2001). *Tau$^{mGFP-LacZ}$* is a Cre recombinase reporter strain having *lox-STOP-lox-mGFP-IRES-NLS-LacZ-pA* integrated into the second exon of the *Tau* locus (Hippenmeyer et al., 2005).

Tamoxifen induction of Cre recombinase was done by interperitoneal injection of pregnant females carrying transgenic embryos at E10.5-E14.5 with 2-3 mg tamoxifen (Sigma, T55648) in sunflower oil per 40g body weight. Transgenic embryos from E10.5 to E18.5 were harvested. Whole E10.5-E13.5 embryos were fixed in 4% paraformaldehyde for 2 hours at 4˚C. For the E14.5-E18.5 embryos, the spinal columns were dissected out immediately, then fixed in 4% paraformaldehyde overnight at 4˚C. After washing in cold PBS, the tissues were cryoprotected in 30% sucrose in PBS for E10.5-E13.5 embryos or 30% sucrose in H_2O for E14.5-E18.5 overnight. P30 spinal cords were collected following Avertin anesthesia and trans-cardiac perfusion with 4% formaldehyde. Dissected out spinal cords were fixed further by immersion in 2% or 4% formaldehyde overnight at 4 ^{o}C, rinsed in PBS, and cryoprotected in 30% sucrose. After being embedded and frozen in OCT, 30 μm cryosections of embryonic or adult spinal cords were collected using a Leica cryostat.

Immunofluorescence and mRNA in situ hybridization

Cryosections from embryonic and adult tissues or free floating sections from adult tissues were incubated with the appropriate dilution of primary antibody in PBS/3% normal donkey serum/0.1% Triton X-100, followed by incubation with the appropriate anti-goat or anti-donkey secondary antibodies conjugated with Alexa 488, 563, 594, or 647 (Molecular Probes). Mouse monoclonal antibodies used were: GFAP (1:400, Sigma-Aldrich, G3893), NeuN (1:1000, Chemicon, MAB377), Glutamine synthetase (1:500, Chemicon), PDGFRα (1:200, BD Biosciences), S100β (1:2000, Sigma), and APC (1:100, Oncogene Sciences, clone CC-1). Rabbit polyclonal antibodies used were: GFP (1:500, Molecular Probes, A6455), Sox2 (1:3000, Chemicon), Pax2 (1:200, Invitrogen), and Olig2 (1:2000, Chemicon). Guinea pig polyclonal antibodies used were: anti-Tlx3 (1:3000, gift T. Muller), anti-Lbx1 (1:10,000, gift T.Muller), and guinea pig anti-Sox10 (1:2000, gift M. Wegner). Chick anti-GFP (1:500, Aves lab) and anti-β-galactosidase (1:2000, Abcam) were also used. Confocal imaging was carried out with a Zeiss LSM510 confocal microscope. For each experiment, multiple sections from at least 3 animals were analyzed.

In situ hybridization was performed as described previously (Birren et al., 1993). Digoxigenin-labeled riboprobes were generated from plasmids containing part of the coding region of each gene: Ascl1 (Johnson et al., 1990), Cre (gift A. Joyner), and Insm1 (gift W. Huttner). The images were photographed using a Zeiss Discovery V12 microscope.

Electron microscopy

After tamoxifen administration at E14.5, P30 *Ascl1*$^{CreERT2/+}$*;R26R-stop-LacZ* mice were perfused with 4% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. Spinal cords (thoracic segments) were dissected out and immersion fixed in the same fixative overnight at 4°C. The tissue was then dissected into 1 nm thick discs and post-fixed with 1% OsO_4, followed by en bloc

stain with 2% uranyl acetate for 15 min and was dehydrated in a graded series of ethanol to 100%, and finally embedded in Poly/bed 812 (Polysciences Inc., Warrington, PA). Thin sections (60 nm) were made by a Leica Ultracut microtome and post-stained with uranyl acetate and lead citrate. The sample grids were examined with a FEI Tecnai transmission electron microscope at 120kV of accelerating voltage, the digital images were captured with an Olympus SIS Morada CCD camera.

RESULTS

Redefinition of the Ascl1^{+} progenitor domain in the neural tube using *Ascl1*$^{CreERT2/+}$ and BAC Tg *Ascl1-CreER*^{TM} mice

In the E11.5 mouse dorsal neural tube, Ascl1 marks a broad progenitor domain where two major neuronal types (inhibitory dIL^{A} and excitatory dIL^{B}) are generated (Mizuguchi et al., 2006; Wildner et al., 2006). In the E11.5 *Ascl1*$^{GFP/+}$ neural tube where GFP was knocked into the *Ascl1* locus, Ascl1-GFP cells co-label with either Pax2, a dIL^{A} neuronal marker, or Tlx3, a dIL^{B} neuronal marker (Fig. 3.1), consistent with a previous study using an independent *Ascl1*$^{GFP/+}$ transgenic line (Wildner et al., 2006). Thus, dIL^{A} and dIL^{B} neurons are generated from an Ascl1 expressing common progenitor domain. However, it is not clear how a common progenitor pool defined by a single bHLH transcription factor can produce two very different neuronal types. Here I divide this Ascl1^{+} progenitor domain into at least two subdomains located apically and basally in the ventricular zone in the neural tube, and show that these two spatially separable Ascl1^{+} populations contribute differentially to inhibitory dIL^{A} and excitatory dIL^{B} neuronal populations.

Retroviral tracing studies in the chick spinal cord suggested a model whereby the two dIL neuronal populations arise from progenitor cells undergoing distinct cell division modes (Wildner et al., 2006). It was shown that dIL^{B} excitatory neurons can be generated from either symmetric or asymmetric divisions, but dIL^{A} neurons arise only from asymmetric cell divisions (Wildner et al., 2006). To probe the lineage relationship between dIL^{A} and dIL^{B} neurons further, two distinct tamoxifen inducible Cre mouse models, an *Ascl1*^{CreERT2} knock-in and a BAC transgenic (Tg) *Ascl1-CreER*^{TM}, were used (Fig. 3.2A-B). In situ hybridization of *Cre* mRNA in E11.5 spinal cord showed the spatially distinct expression of *Cre* in the Ascl1 progenitor domain. *Cre* from *Ascl1*$^{CreERT2/+}$

embryos appears to reliably mimic the endogenous *Ascl1* pattern whereas *Cre* expression from the BAC Tg *Ascl1-CreER*^{TM} is enriched at the lateral edge of progenitor domain, the domain that contains cells transitioning to the differentiated state (Fig. 3.2C-E). Indeed, the pattern of *Cre* expression from the BAC Tg *Ascl1-CreER*^{TM} line is more similar to that of *Insm1*, a known marker of progenitor cells undergoing symmetrical terminal divisions in the telencephalon (Fig. 3.2F) (Duggan et al., 2008; Farkas et al., 2008). Thus, tracing Ascl1 progenitor fates using the two temporally discrete Cre lines provides additional detailed insights into Ascl1 lineage cells and how they are generated.

Two spatially and temporally distinct Ascl1^{+} progenitors contribute differentially to inhibitory dIL^{A} and excitatory dIL^{B} neuronal populations in the dorsal spinal cord

To label and follow the fate of Ascl1^{+} progenitors marked by *Ascl1*^{CreERT2} or BAC Tg *Ascl1-CreER*^{TM} in the dorsal neural tube, tamoxifen was administered to pregnant dams at day 11.5 post coitum also carrying the *R26R-stop-YFP* allele. In both Cre models harvested 24 hours after tamoxifen administration, the position of YFP^{+} cells along the dorsal ventral axis of the neural tube, and their overlap with Lbx1, a common dIL neuronal marker, demonstrates that the labeled cells are transitioning from Ascl1^{+} progenitors to differentiated dIL neurons (Fig. 3.3A-B' and data not shown). Essentially all YFP^{+} cells in BAC Tg *Ascl1-CreER*^{TM}*;R26R-stop-YFP* embryos are located in the lateral mantle zone, suggesting Ascl1^{+} progenitors captured by this transgenic line differentiate rapidly after Cre recombination (Fig. 3.3B'). This is consistent with the enrichment of Cre expression in the lateral edge of progenitor domain (Fig. 3.2E). In *Ascl1*$^{CreERT2/+}$*;R26R-stop-YFP* embryos, although many YFP^{+} cells are located in the differentiating intermediate or mantle zone, a few YFP^{+} cells remain in the progenitor domain, and thus are not terminally differentiated (Fig. 3.3B). Indeed,

a few YFP^+ cells still remain in the ventricular zone even three days after tamoxifen administration (data not shown). This indicates that some $Ascl1^+$ cells labeled by the *Ascl1*^{CreERT2} line are earlier in the lineage than those labeled by *Ascl1-CreER*^{TM} and thus must be located in an apical subdomain in the ventricular zone (Fig. 3.3G).

It is clear from this initial characterization that the two Ascl1-Cre models are labeling Ascl1 progenitors in slightly different stages of development in the neural tube. To determine if there are differences in the fates of these progenitor populations, E17.5 spinal cords were harvested after tamoxifen administration at E11.5. Most YFP^+ cells in both lines become neurons, not glia as determined by co-localization with NeuN but not Olig2 (Fig. 3.3C-C' and data not shown), demonstrating $Ascl1^+$ progenitors at E11.5 are neuronal lineage restricted (Battiste et al., 2007). Neuronal subtypes were analyzed with Pax2, the dIL^A inhibitory neuronal marker, and Tlx3, the dIL^B excitatory neuronal marker. Whereas the *Ascl1*$^{CreERT2/+}$ line captures both $Pax2^+$ dIL^A and $Tlx3^+$ dIL^B neurons efficiently, the BAC Tg *Ascl1-CreER*^{TM} line preferentially marks only $Tlx3^+$ dIL^B neurons (Fig. 3.3E-H). One interpretation is that the BAC Tg *Ascl1-CreER*^{TM} captures only the later stage of Ascl1 progenitor that is in its terminal symmetric division, a division that is restricted to the dIL^B, excitatory neuron fate. This line misses the earlier stage $Ascl1^+$ progenitors that can generate dIL^A neurons from asymmetric divisions. In contrast, the *Ascl1*$^{CreERT2/+}$ line marks all Ascl1 domains (Fig. 3.2D), and thus both dIL^A and dIL^B neuronal populations, regardless of division pattern, are detected in the lineage. Interpreting these data in the context of the earlier retroviral tracing studies, we propose that there are Ascl1 expressing progenitors at multiple closely related stages that are restricted to particular cell fates (Fig. 3.3G).

Cell autonomous requirement of Ascl1 in differentiation and cell type specification in the late phase of neurogenesis

Functions of Ascl1 in neuronal differentiation and subtype specification in the dIL lineage have been demonstrated from analyses of the *Ascl1* null mutant (Mizuguchi et al., 2006; Wildner et al., 2006). To achieve more refined functional analysis of Ascl1 in dIL^A and dIL^B lineage development, avoiding earlier effects of the loss of Ascl1 in the progenitor domain, I took advantage of a conditional allele of *Ascl1* (*Ascl1*^{fl}) generated by the laboratory of our collaborator Francois Guillemot. Combining this conditional allele with tamoxifen inducible Cre alleles allows the phenotype of an individual mutant cell in a field of wildtype cells to be determined.

The design of the *Ascl*1 floxed allele is shown in Fig. 3.4. The allele was designed such that in the presence of Cre, the coding exon of *Ascl1* gets floxed out as the coding region for the fluorescent marker Venus is shifted in frame. To validate whether the conditional mutant allele of *Ascl1* works as designed, *Pax3*$^{Cre/+}$*;Ascl1*$^{fl/fl}$ embryos were harvested at E11.5 (Fig. 3.5). Since Cre expression in this transgenic line is restricted in the dorsal region of the neural tube (Fig. 3.5 inset) (Engleka et al., 2005), Ascl1 is ablated only in the dorsal domain whereas Ascl1 in the ventral domain remains intact (Fig. 3.5B''). Furthermore, Venus is detected only in the dorsal half of the neural tube (Fig. 3.5A-A' and B-B'). These results demonstrate the precise control of the *Ascl1* conditional allele is possible through the use of different Cre driver lines. The expression of *Venus* turned out to be inefficient and inconsistent in the multiple experiments, thus, a Cre reporter gene such as *R26R-stop-YFP* was used in the following studies to track the cells where cre-flox recombination occurs.

To specifically ablate Ascl1 in progenitors at the late phase of neurogenesis (E11.5-13), bypassing complications of loss of Ascl1 in early neurogenesis (E10-11.5), tamoxifen was administered at E11.5 to *Nestin-*

*CreER^{T2};R26R-stop-YFP (*or *LacZ);Ascl1$^{fl/\Delta}$* (*Ascl1^{cKO}*) or *Nestin-CreER^{T2};R26R-stop-YFP (*or *LacZ);Ascl1$^{fl/+}$* (control) embryos (Fig. 3.6). Nestin is a marker for neural stem cells and is expected to appear prior to Ascl1 (Wiese et al., 2004), supporting *Nestin-CreER^{T2}* as a suitable Cre model to ablate *Ascl1*. The tamoxifen treated embryos were harvested at E13.5, and the most notable phenotype is that more YFP^{+} (LacZ^{+}) cells are present in the ventricular zone in the *Ascl1^{cKO}* embryos than in the controls (Fig. 3.6A-B'). However, there are approximately the same number of YFP^{+} cells in the mantle zone in both the *Ascl1^{cKO}* and control embryos (Fig. 3.6A-B'). The increase in YFP^{+} cells in the VZ suggests *Ascl1* deficient neuronal progenitors aberrantly continue proliferating rather than differentiating. This phenotype demonstrates the cell autonomous activity of Ascl1 in inducing neuronal differentiation of progenitor cells.

The requirement of Ascl1 for dIL^{A} neurons has been assessed using the *Ascl1* null mutant (Mizuguchi et al., 2006; Wildner et al., 2006). However, since Ascl1 not only functions cell autonomously, but also modulates Notch signaling in adjacent cells in a non-cell autonomous manner, it has not been directly shown through which mechanism Ascl1 regulates neuronal subtype specification. To analyze the cell autonomous function of Ascl1 in neuronal subtype specification, the *Tau$^{mGFP\text{-}LacZ}$* reporter line was used in combination with the *Ascl1^{cKO}* to follow cells that had lost Ascl1. After tamoxifen administration at E11.5, E17.5 spinal cords were harvested from *Nestin-CreER^{T2};Tau$^{mGFP\text{-}LacZ}$;Ascl1$^{fl/\Delta}$* (*Ascl1^{cKO}*) or *Nestin-CreER^{T2};Tau$^{mGFP\text{-}LacZ}$;Ascl1$^{fl/+}$* (control) embryos (Fig. 3.6C). In *Ascl1 ^{cKO}* spinal cords, the percentage of β-gal^{+} neurons that became dIL^{A} was decreased, whereas the percentage that became dIL^{B} neurons was increased concomitantly (Fig. 3.6D-E). These results show that Ascl1 biases cells towards a dIL^{A} neuronal fate in a cell autonomous manner. In addition, the *Ascl1* deficient neuronal progenitors may instead adopt a dIL^{B} fate.

At late stages of embryogenesis Ascl1 marks glial progenitor cells prior to their specification to oligodendrocytes or astrocytes

Ascl1^{+} progenitors in the spinal cord at late embryonic stages (E14-E18) give rise to glia. Previous studies suggested that Ascl1^{+} glial precursors are restricted to the oligodendrocyte lineage (Battiste et al., 2007; Parras et al., 2007; Sugimori et al., 2007; Sugimori et al., 2008). This conclusion was based on in vitro culture studies where Ascl1 was ectopically expressed in neural progenitor cells (Parras et al., 2004; Sugimori et al., 2007), or on fate mapping of Ascl1 cells using the BAC Tg *Ascl1-CreER^{TM}* mice (Battiste et al., 2007). Given that the *Ascl1^{CreERT2}* knock-in seemed to more reliably mark all *Ascl1* lineage cells, we used this model to examine the gliogenic lineage in the spinal cord in more detail. In the E14.5 mouse spinal neural tube, Ascl1 is not restricted to the ventricular zone but is found spread out throughout the spinal cord (Fig. 3.7B). This pattern is comparable with another progenitor cell marker Sox2 (Fig. 3.7B-B''). Indeed, throughout the spinal cord, a vast majority of *Ascl1* expressing cells co-express Sox2, and thus, defines these cells as progenitors (Fig. 3.7C-C'''). Notably, in the dorsal ventricular zone, many Ascl1 expressing cells do not express Olig2 or PDGFRα, early markers of the oligodendrocyte lineage, suggesting Ascl1^{+}/Olig2^{-}/PDGFRα^{-} cells are progenitors at a stage prior to oligodendrocyte fate specification (Fig. 3.7D,E). In contrast, in the ventral ventricular zone, and in grey and white matter, most of Ascl1^{+} cells co-express Olig2 or PDGFRα, suggesting they are oligodendrocyte precursors (Fig. 3.7D'-E',D''-E'',D'''-E''').

Ascl1 lineage cells give rise to not only oligodendrocytes but also astrocytes

To fate map *Ascl1* lineage cells at late stages of embryogenesis, tamoxifen was administered to pregnant transgenic dams carrying E14.5 *Ascl1$^{CreERT2/+}$;R26R-stop-YFP* or BAC Tg *Ascl1-CreER^{TM} ;R26R-stop-YFP* embryos, and spinal cords from the resulting P30 offspring were harvested (Fig. 3.8A-B). In both models,

Ascl1^{+} progenitor cells marked at E14.5 are restricted to glial lineages and do not give rise to neurons (data not shown). Surprisingly, Ascl1 lineage cells from *Ascl1$^{CreERT2/+}$;R26R-stop-YFP* give rise not only to Sox10^{+}, Olig2^{+}, CC-1^{+} oligodendrocytes (Fig. 3.8Aa-Ab', and data not shown) but also to GFAP^{+}, Glutamine synthetase^{+}, S100β^{+} astrocytes (Fig. 3.8Ac-Ac', and data not shown). In contrast, only oligodendrocytes are derived from progenitors marked in the BAC Tg *Ascl1-CreER^{TM}* line (Fig. 3.8Ba-Bc') (Battiste et al., 2007). Thus, Ascl1 expressing progenitor populations in the E14.5/E15.5 spinal neural tube are restricted to the glial lineage but can generate both oligodendrocytes and astrocytes, similar to the fate of Nestin expressing cells (*Nestin-CreER^{T2};R26R-stop-YFP*, Fig. 3.8C-Cc').

Dorsally derived oligodendrocytes labeled by Ascl1 are genetically different from Ascl1^{-}/Olig2^{+} oligodendrocytes originating from the ventral domain (Fig. 3.9) (Cai et al., 2005; Vallstedt et al., 2005). It still remains unanswered whether this late developing population of oligodendrocytes mature into myelinating oligodendrocytes. Electron micrographs of P30 spinal cord from *Ascl1$^{CreERT2/+}$;R26R-stop-LacZ* demonstrate that Ascl1 lineage oligodendrocytes generated at the late embryonic stages are myelinating (Fig. 3.8D). In addition, β-gal labeled astrocytes were identified by their ultrastructural features (Fig. 3.8E).

Ascl1 is required for oligodendrocyte development in a cell autonomous manner

Previous studies showed the decrease of oligodendrocyte specification markers such as Olig2 or Nkx2.2 in the spinal cord, forebrain, and cerebellum in the *Ascl1* null mutant (Parras et al., 2007; Sugimori et al., 2008; Grimaldi et al., 2009). Neurosphere cultures from *Ascl1* null mutants had a decreased ability to generate oligodendrocytes relative to neurospheres from control animals (Parras et al., 2004; Sugimori et al., 2007; Sugimori et al., 2008). These results suggest Ascl1 is

a key player in oligodendrogenesis. However, interpretation of these phenotypes is complicated by the fact that the progenitor domains at stages earlier than gliogenesis are disrupted in the *Ascl1* null mutant. In addition, since *Ascl1* nulls are neonatal lethal, the role of Ascl1 in oligodendrocyte development that continues through postnatal stages has been impossible to assess. To obviate these complications, I investigated the in vivo function of Ascl1 in gliogenesis using the conditional allele of *Ascl1*. *Glast*^{CreERT2} is an inducible Cre knock-in strain where CreERT2 is inserted in the locus of GLAST (Mori et al., 2006). *Nestin-CreER*^{T2} or *Glast*$^{CreERT2/+}$ was used as Cre driver to flox out *Ascl1* after E14.5 when neurogenesis is complete. In contrast to the lethality of *Ascl1* null mice at birth (Guillemot et al., 1993), the Ascl1 cKO mice survive postnatally and do not show any severe gross behavioral phenotypes at least until P13. P13 spinal cords of *Nestin-CreER*^{T2}*;R26R-stop-YFP; Ascl1*$^{fl/\Delta}$ were harvested to examine how the YFP^{+} cells developed postnatally after conditional ablation of Ascl1 (Fig. 3.10). In control *Nestin-CreER*^{T2}*;R26R-stop-YFP* embryos after E14.5 tamoxifen treatment, YFP^{+} cells co-label with markers for both astrocytes and oligodendrocytes throughout the spinal cord (Fig. 3.10B). However, in the Ascl1 cKO spinal cord, there is a specific loss of cells with YFP^{+} and oligodendrocyte markers co-localized (90±19.7 (control) versus 35.6±10.6 (cKO) /section) (Fig. 3.10A,C). In contrast, there is no loss of YFP^{+} astrocyte cells (82.5±3.5 (control) versus 77 ±10.6 (cKO) /section) (Fig. 3.10C). Taken together, these results demonstrate that although Ascl1 is present in progenitors to both astrocytes and oligodendrocytes, it is only required in the oligodendrocyte lineage to generate normal numbers of this specific cell type.

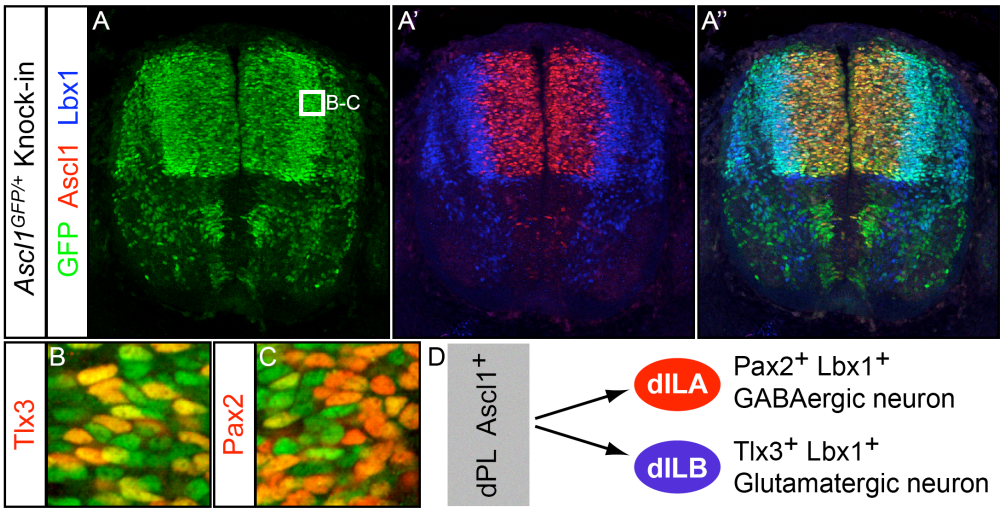


Figure 3.1. Ascl1^{+} progenitors give rise to both dIL^{A} and dIL^{B} neurons in the dorsal spinal cord.
(A-A'') Images from immunofluorescence for GFP (green), Ascl1 (red), and Lbx1 (blue) in *Ascl1*$^{GFP/+}$ knock-in mouse spinal neural tube at E12.5. (B-C) Ascl1-GFP expressing cells co-label with inhibitory dIL^{A} neuronal marker Pax2 (B) and excitatory dIL^{B} neuronal marker Tlx3 (C) in the lateral region of neural tube. (D) dIL^{A} and dIL^{B} neurons are generated from an Ascl1 expressing common progenitor domain.

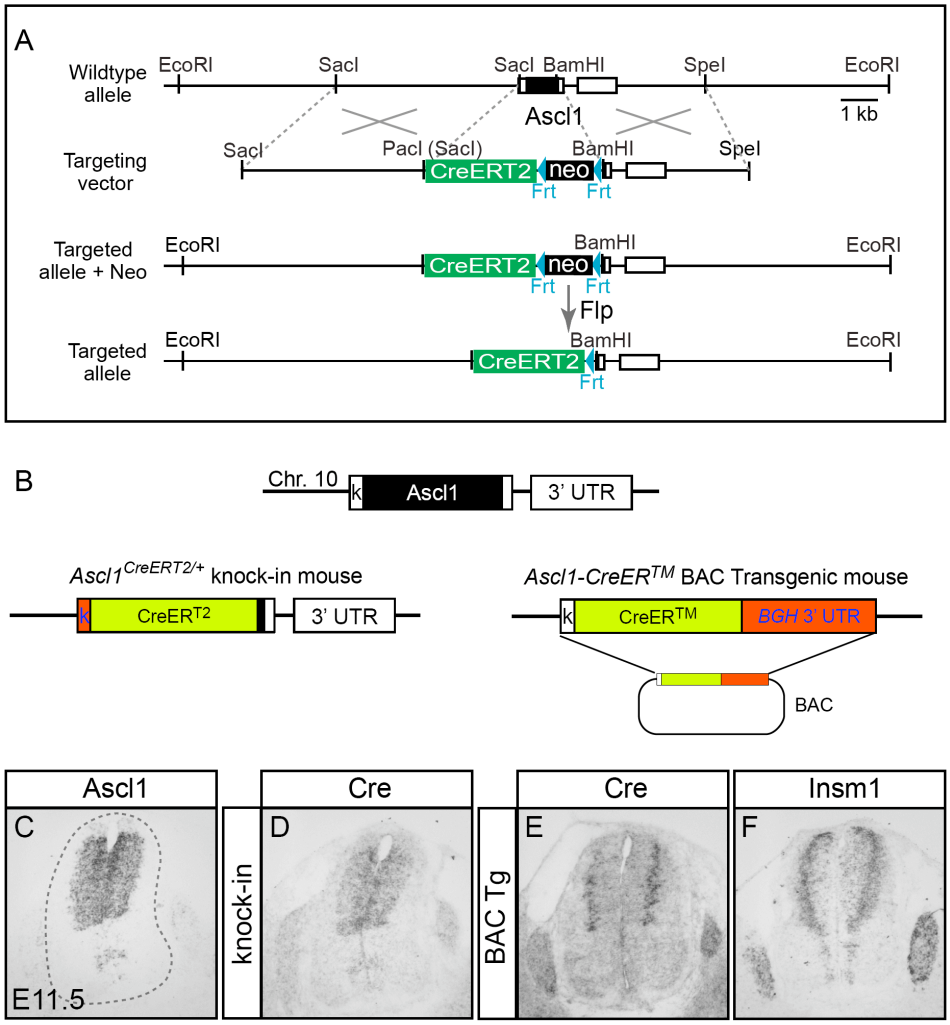


Figure 3.2. *Ascl1*^{CreERT2} knock-in and *Ascl1-CreER*^{TM} BAC transgenic mice mark Ascl1 expressing progenitors at temporally distinct stages.
(A) Schematic illustration of *Ascl1*$^{CreERT2/+}$ knock-in mouse generation strategy. (B) Comparison of *Ascl1*$^{CreERT2/+}$ knock-in and *Ascl1-CreER*^{TM} BAC transgenic (Tg) mouse designs. Knock-in line contains extra short-linker upstream of endogenous kozak sequence (k). In BAC Tg line, endogenous 3' UTR is replaced by the bovine growth hormone (BGH) 3' UTR (Battiste et al., 2007). (C-F) Distinct pattern of Cre mRNA expression for knock-in and BAC Tg mice in E11.5 spinal neural tube. The knock-in line appears to express Cre in an earlier progenitor stage than that seen in the BAC Tg line. Cre from the knock-in (D) appears to faithfully mimic the endogenous Ascl1 pattern (C). In contrast, the expression from the BAC Tg (E) enriched at the lateral edge of progenitor

domains containing more differentiated cells is more comparable with the of Insm1, a marker of progenitor cells undergoing symmetrical terminal divisions in the telencephalon. Abbr: BGH, bovine growth hormone; k, kozak sequence; UTR, untranslated region.

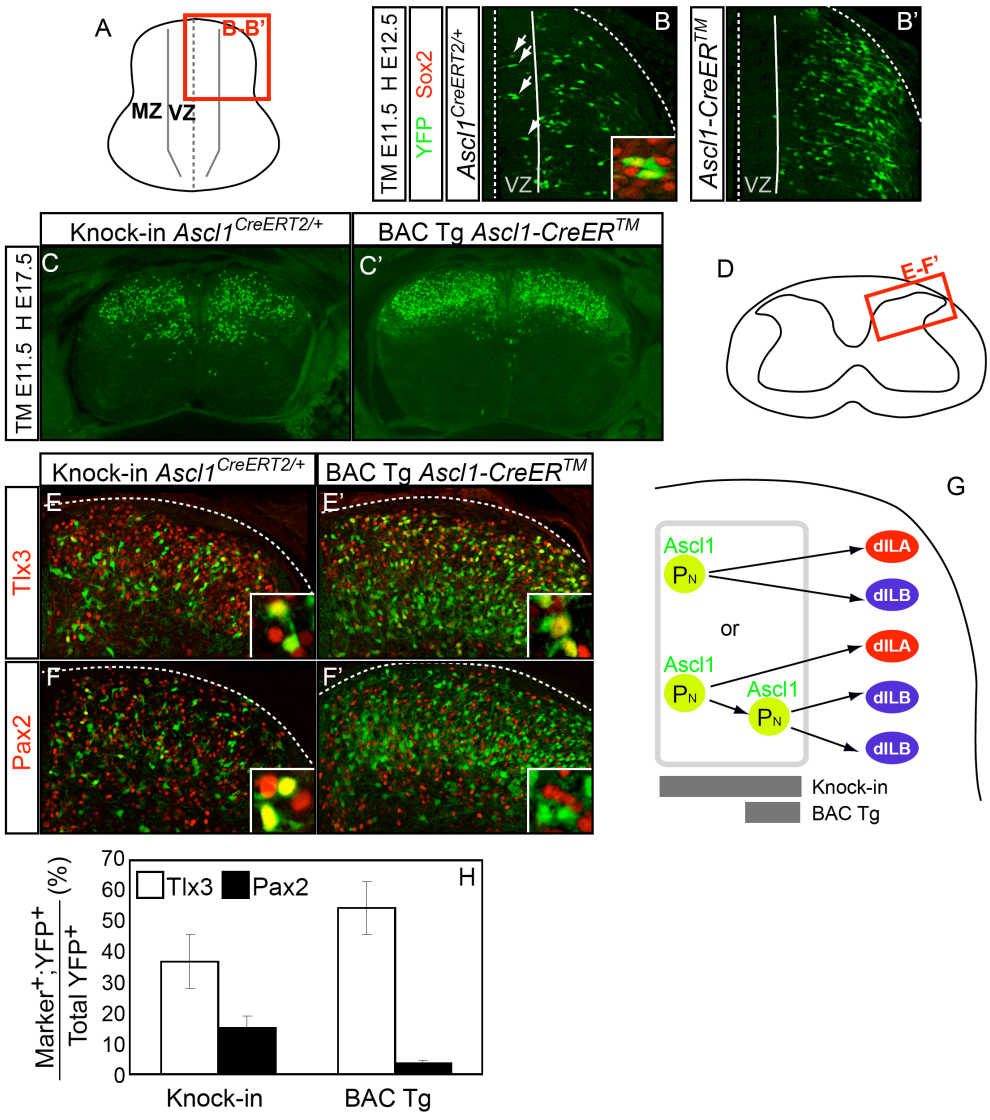
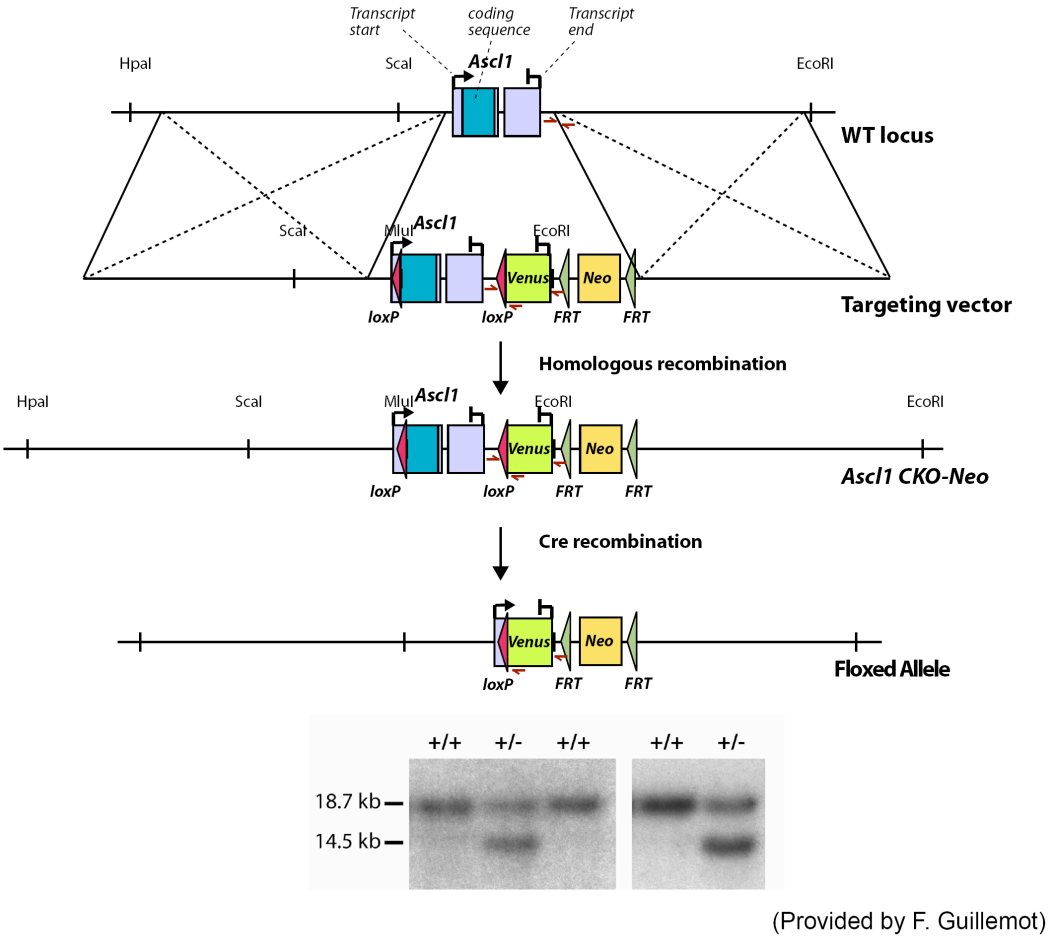


Figure 3.3. The fate mapping of Ascl1 neuronal lineage: *Ascl1*^{CreERT2} for dIL^{A} and dIL^{B} versus BAC Tg *Ascl1-CreER*^{TM} for only dIL^{B}.

(A) Schematic drawing of E12.5 neural tube. (B-B') Immunofluorescence images of E12.5 neural tubes of *Ascl1*$^{CreERT2/+}$*;R26R-stop-YFP* or *Ascl1-CreER*^{TM}*;R26R-stop-YFP* after the tamoxifen administration at E11.5. (B) Some YFP^{+} in *Ascl1*$^{CreERT2/+}$ embryos are present in the ventricular zone (arrows), colabeling Sox2 (inset) (B') Most of, if not all, YFP^{+} cells in *Ascl1-CreER*^{TM} embryos differentiate into the mantle zone. (C-F') Immunofluorescence images of E17.5 neural tubes of *Ascl1*$^{CreERT2/+}$*;R26R-stop-YFP* or *Ascl1-CreER*^{TM}*;R26R-stop-YFP* after the tamoxifen administration at E11.5. In both

transgenic line, YFP^+ give rise to neurons in the dorsal horn of the spinal cord (C-C'). (D) Schematic drawing of E17.5 spinal cord. (E-F') YFP^+ cells in *Ascl1*$^{CreERT2/+}$*;R26R-stop-YFP* colabel both Pax2 and Tlx3, inhibitory dIL^A neuronal marker and excitatory dIL^B neuronal marker, respectively. In contrast, YFP^+ cells in *Ascl1-CreER*^{TM}*;R26R-stop-YFP* label only Tlx3 (F'). (G) Lineage model for generation of dIL^A and dIL^B neurons in the dorsal neural tube. (H) Quantification of the percentage of YFP cells co-labeled with the Pax2 or Tlx3 in *Ascl1*^{CreERT2} and *Ascl1-CreER*^{TM} spinal cord. Abbr: MZ, mantle zone; P_N, neuronal progenitor; VZ, ventricular zone.



(Provided by F. Guillemot)

Figure 3.4. Generation of the conditional knockout of Ascl1 (*Ascl1^{fl}*).
Homologous recombination inserted one loxP site at the start codon of *Ascl1*. A second loxP site is located at the 3' end of the transcript (following the polyA signal) and is immediately followed by a Venus cassette. A neo selection gene, flanked by FRT sites, was inserted 3' of *Ascl1*. Upon Cre recombination, the entire coding sequence and 3' UTR of *Ascl1* is deleted and the expression of the Venus cassette placed under the control of *Ascl1* upstream regulatory elements. ES clones were screened by Southern using EcoRI digested DNA. The wt band is 18.7 kb and the targeted allele is 14.5 kb. Additional Southern analysis demonstrated the integrity of the 3' arm (data not shown). Blastocyst injection with the targeted ES cells resulted in germline chimeras. This figure is provided by F. Guillemot.

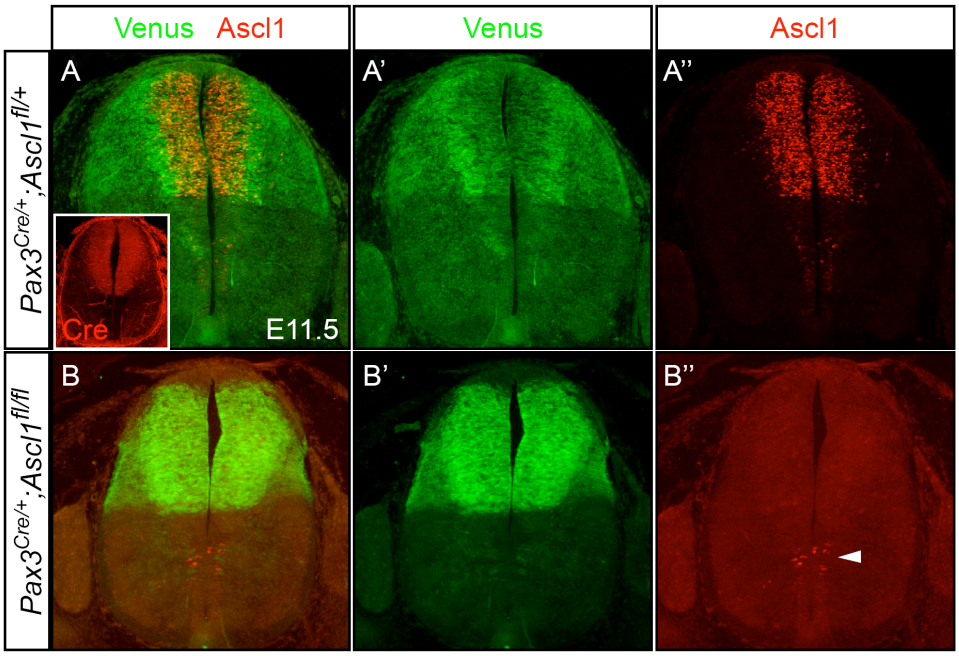


Figure 3.5. Specific ablation of Ascl1 in the dorsal neural tube of *Pax3*$^{Cre/+}$;*Ascl1*$^{fl/fl}$. Immunofluorescence images of E11.5 *Pax3*$^{Cre/+}$;*Ascl1*$^{fl/+}$ (A-A'') and *Pax3*$^{Cre/+}$;*Ascl1*$^{fl/fl}$ (B-B''). As Cre expression is restricted in the dorsal region of neural tube of *Pax3*$^{Cre/+}$ (inset of A), Ascl1 expression is deleted in the dorsal progenitor domain (B'), whereas Ascl1 expression is still detected in the ventral progenitor domain (arrow). After deleting out *Ascl1* by Cre recombination, Venus is expressed in the cells instead (A-A', B-B').

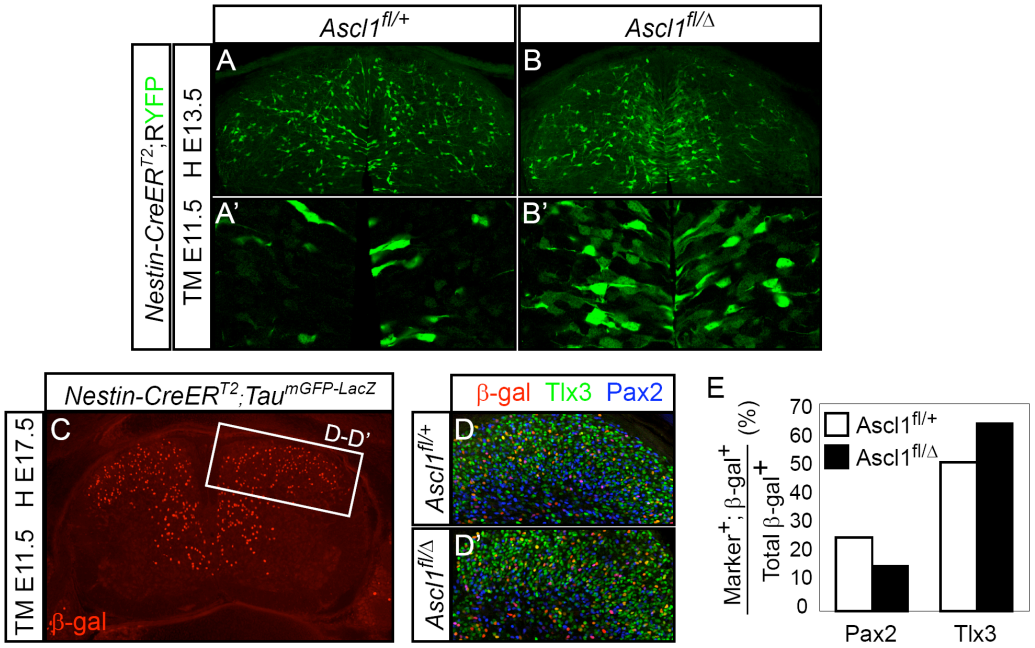


Figure 3.6. Ascl1 is required for neuronal differentiation and dIL^{A} neuron specification.
(A-B') Immunofluorescence images of E13.5 neural tubes of *Nestin-CreER^{T2};R26R-stop-YFP* carrying *Ascl1$^{fl/+}$* or *Ascl1$^{fl/\Delta}$* after the tamoxifen administration at E11.5. Much more YFP^{+} cells persist in the ventricular zone when *Ascl1* is ablated conditionally (B, B' for the magnified view). (C-D') Immunofluorescence images of E17.5 spinal cord of *Nestin-CreER^{T2};Tau$^{mGFP-LacZ}$* carrying *Ascl1$^{fl/+}$* or *Ascl1$^{fl/\Delta}$* after the tamoxifen administration at E11.5. (C) The transverse section of E17.5 spinal cord of *Nestin-CreER^{T2};Tau$^{mGFP-LacZ}$* showing β-gal^{+} neuronal lineage cells. (D-D') Immunofluorescence images showing Pax2^{+} (blue) dIL^{A} neurons and Tlx3^{+} (green) dIL^{B} neurons among β-gal^{+} neuronal lineage in *Ascl1$^{fl/+}$* (D) or *Ascl1$^{fl/\Delta}$* background (D'). (E) Quantification of the percentage of β-gal^{+} cells co-labeled with the Pax2 or Tlx3 in E17.5 *Ascl1$^{fl/+}$* and *Ascl1$^{fl/\Delta}$* spinal cords.

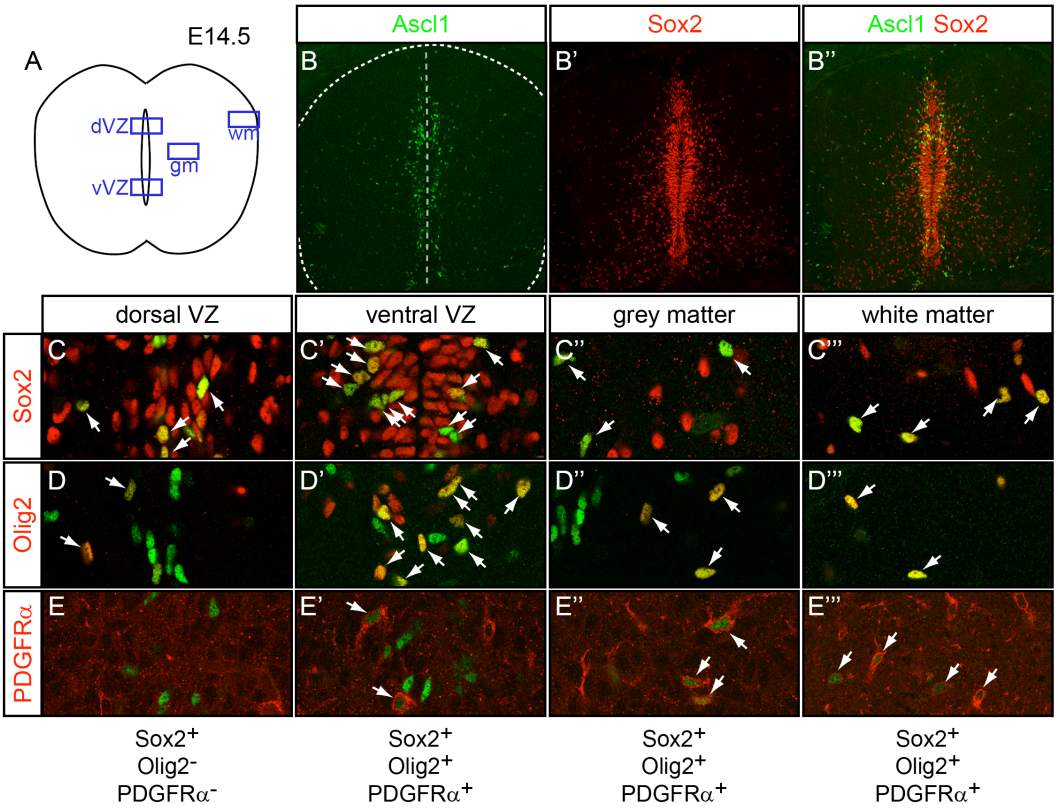


Figure 3.7. Ascl1 expression in the spinal neural tube at the late stage of embryogenesis.
(A) Illustration showing E14.5 mouse neural tube with the panels of demarcation for each subregions. (B-B'') Immunofluorescence showing Ascl1 (green) and Sox2 (red), a progenitor cell marker, in the E14.5 spinal cord. Ascl1 expression is enriched in the ventricular zone as well as grey and white matters. (C-C''') Most, if not all, Ascl1 expressing cells are co-labeled with Sox2 (red) throughout the neural tube. (D-E''', arrows) Many Ascl1^{+} progenitors express Olig2 (D'-D''', arrows) and PDGFRα (E'-E''', arrows) in the ventral ventricular zone, grey and white matter, whereas few cells express Olig2 (D) or PDGFRα (E) in the dorsal ventricular zone. Abbr: dVZ, dorsal ventricular zone; gm, grey matter; vVZ, ventral ventricular zone; wm, white matter.

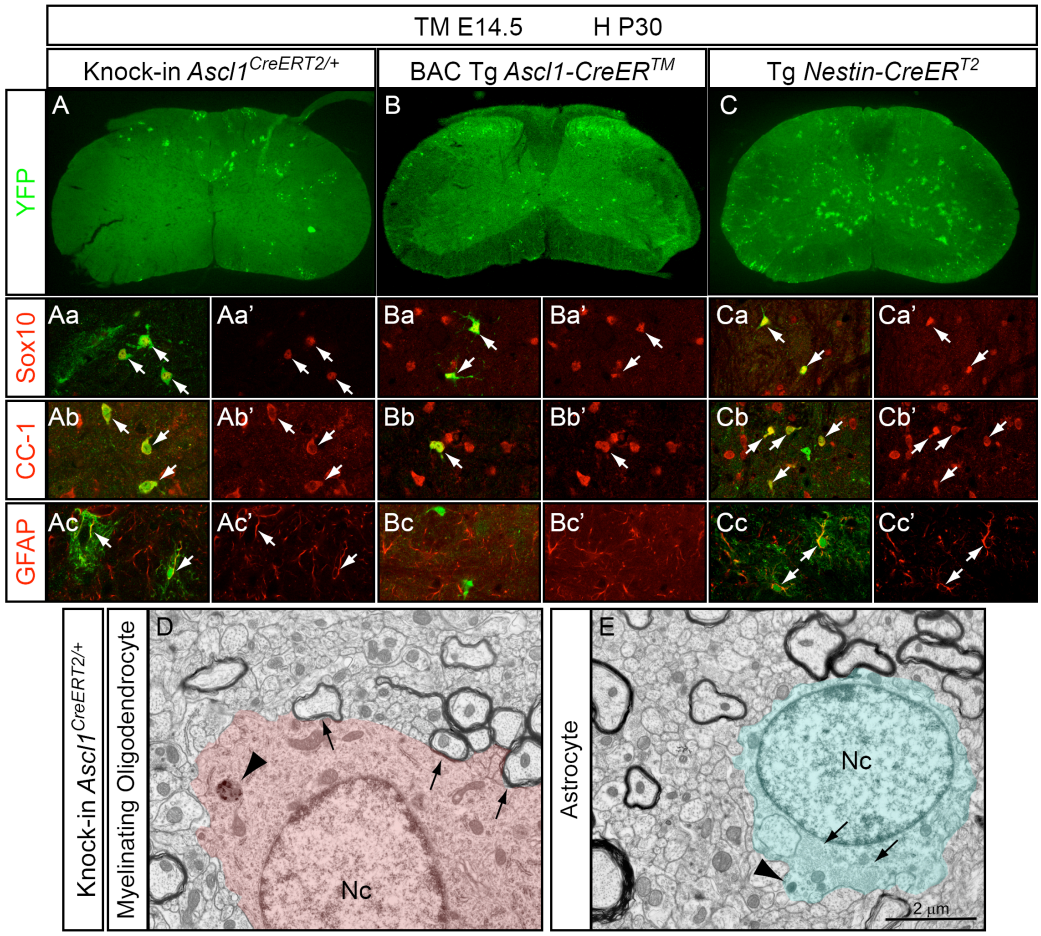


Figure 3.8. Ascl1 lineage cells marked at E14.5 give rise to oligodendrocytes and astrocytes.

(A-C) Long term fate mapping of YFP^{+} lineage cells in the P30 spinal cord from three different transgenic lines: *Ascl1*$^{CreERT2/+}$ (A), BAC Tg *Ascl1-CreER*^{TM} (B), and *Nestin-CreER*^{T2} (C) crossed with *R26R-stop-YFP* reporter lines. Tamoxifen was administered at E14.5 to lineage trace the progenitors at the late stage of embryogenesis. (Aa-Cc') Double immunofluorescence staining of YFP^{+} lineage cells with Sox10 (oligodendrocyte marker), CC-1 (mature oligodendrocyte marker), and GFAP (astrocyte marker). Ascl1 derived cells marked by *Ascl1*$^{CreERT2/+}$ give rise to both mature oligodenrcotyes (Aa-Ab') and astrocytes (Ac-Ac'). In contrast, Ascl1 derived cells marked by *Ascl1-CreER*^{TM} BAC Tg give rise to only oligodendrocytes (Ba-Bb'), not astrocytes (Bc-Bc'). Fate mapping of glia progenitors marked by *Nestin-CreER*^{T2} showed both oligodenrocytes and astrocytes (Ca-Cc'). (D-E) For ultrastructural analysis of glia lineage from Ascl1^{+} progenitor, tamoxifen was administered to *Ascl1*$^{CreERT2/+}$*;R26R-stop-LacZ* embryos at E14.5 and LacZ^{+} Ascl1 lineage cells were examined at P30 spinal cord by electron microscopy.

$LacZ^{+}$ Ascl1 lineage cells (arrowhead) become the mature oligodendrocytes with myelin (arrows), and astrocytes (arrows). Abbr: Nc, nucleus. Electron micrographs were imaged by X. Liu.

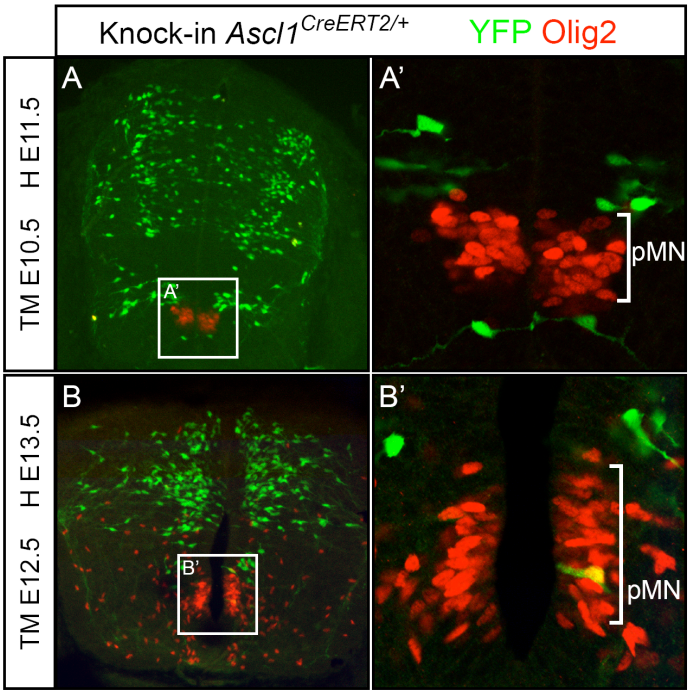


Figure 3.9. Oligodendrocytes generated from early embryonic stages are not Ascl1 lineage.
Immunofluorescence images of *Ascl1*$^{CreERT2/+}$*;R26R-stop-YFP* neural tube sections harvested 24 hours after tamoxifen injection at E10.5 (A-A') or at E12.5 (B-B'). YFP^{+} cells (green) are not derived from pMN domain where early oligodendrycte precursors reside and do not express Olig2 (red).

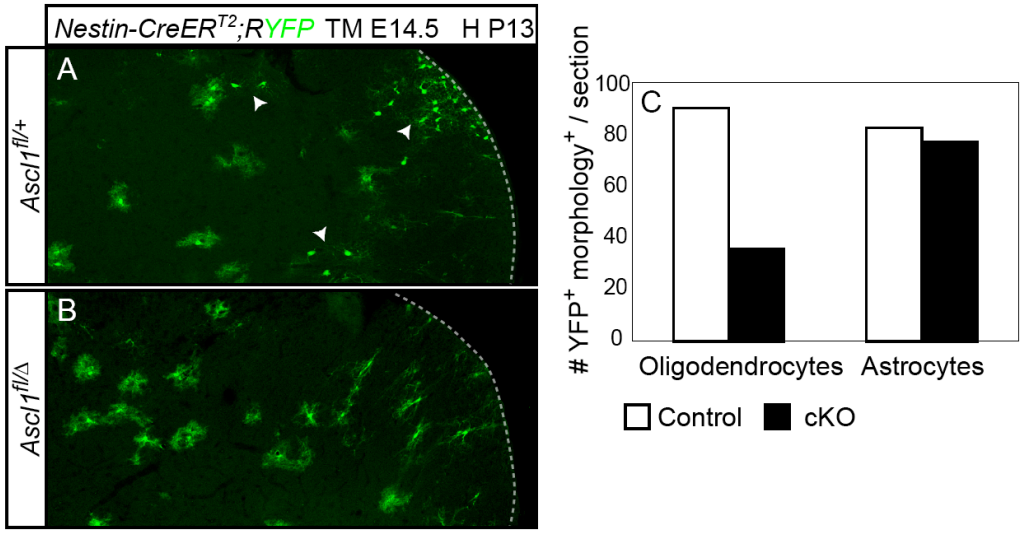


Figure 3.10. Functional analysis of Ascl1 for gliogenesis.
(A-B) Immunofluorescence images showing YFP$^+$ cells from P13 spinal cord of *Nestin-CreER^{T2};R26R-stop-YFP;Ascl1$^{fl/+}$* or *Ascl1$^{fl/\Delta}$* after tamoxifen treatment at E14.5. YFP$^+$ oligodendrocytes are not detected in *Ascl1$^{fl/\Delta}$* spinal cord (B), whereas both YFP$^+$ astrocytes and oligodendrocytes (arrowheads) are detected in *Ascl1$^{fl/+}$* spinal cord (A). (C) Quantification of the number of YFP$^+$ cells having oligodendrocyte or astrocyte-like morphology in P13 *Ascl1$^{fl/+}$* and *Ascl1$^{fl/\Delta}$* spinal cords.

DISCUSSION

The ability to permanently label progenitor cells at distinct phases of their development has allowed me to gain insight into lineage relationships of distinct subtypes of neurons and glia in the developing nervous system. In the dorsal neural tube, Ascl1 defines neuronal or glial restricted progenitors that are separated temporally during embryogenesis. Ablation of Ascl1 in different developmental contexts suggest in each lineage results in a reduction of only one subtype of cell within that lineage, such as loss of dIL^A but not dIL^B neurons, and loss of oligodendrocytes but not astrocytes. Thus, Ascl1 is an essential factor for correct cell fate decisions at multiple nodes of lineage development in the vertebrate nervous system.

Comparative genetic fate mapping using *Ascl1^{CreERT2}* and BAC Tg *Ascl1-CreER^{TM}*

The *Ascl1^{CreERT2}* knock-in and the *Ascl1-CreER^{TM}* BAC transgenic mouse line both mark Ascl1 lineages throughout the animal but the BAC transgenic line is more restricted in certain lineages: during spinal cord development it can mark the dIL^B neurons but not the dIL^A as the knock in does, and it can label the oligodendrocyte lineage but not astrocytes. The subpopulations labeled by the BAC Tg line appear to be generated from $Ascl1^+$ progenitors that are at slightly later stages of development within the specific lineage. Consistent with this interpretation, in the two neurogenic niches of the adult brain, the subventricular and subgranular zones, the cells labeled by *Ascl1^{CreERT2}* include early stem like cells such as Type 1 or B cells, whereas the cells labeled by the BAC Tg are restricted to a later stage progenitor such as the Type 2 or transit amplifying cells (Kim et al., 2007, see also chapter five). Why are these two mouse models marking temporally distinct $Ascl1^+$ populations? *Ascl1^{CreERT2}* is a knock-in

designed such that the *Ascl1* coding region in one allele is replaced by the inducible Cre recombinase (*CreER*^{T2}), maintaining 5' and 3' UTR essentially unaltered. This results in a *Cre* expression pattern that perfectly mimics the endogenous *Ascl1* expression in every tissue we have examined (Fig. 3.2C-D and data not shown). In contrast, in the BAC Tg *Ascl1-CreER*^{TM} line, the *Ascl1* coding region is replaced by *CreER*^{TM} in a BAC containing 98 kb 5' and 206 kb 3' flanking genomic region (Battiste et al., 2007). In addition, the endogenous 3' UTR was replaced by the heterologous bovine growth hormone 3' UTR. One or more of these differences in design must account for the subtle differences in temporal and spatial control observed with this mouse model. Nevertheless, comparing the lineages marked in each of these Ascl1-Cre mouse models allowed the unique opportunity to investigate neuronal and glial lineage development teasing out a discrete temporal component in vivo.

Ascl1 lineage development in neurogenesis

Unlike the distinct progenitor domains in the stripe-like patterns along the dorsoventral axis of the neural tube at the early phase of neurogenesis (E10-E11.5), the common progenitor domain where Ascl1 positive and negative progenitors are intermingled in the salt-and-pepper pattern generates both dIL^{A} and dIL^{B} at the late phase of neurogenesis (E11.5-E13). Although Ascl1^{+} progenitor populations can give rise to both dIL^{A} and dIL^{B} neurons, the cell division modes and potentials to become dIL^{A} or dIL^{B} neurons are not equipotent for each progenitor (Wildner et al., 2006). My studies suggest that the common Ascl1^{+} progenitor domain is divided into at least two subdomains (apical and basal) that have predictive cell fates. The progenitors generating dIL^{A} neurons from asymmetric cell divisions are located in the apical subdomain where only *Ascl1*^{CreERT2} can label the progenitors. In contrast, the basal subdomain that likely undergoes only symmetric cell divisions, is specifically fated to dIL^{B} neurons. In

vivo time lapse imaging of the radial glia/neural progenitors showed that the early asymmetric divisions in the ventricular zone (apical) precedes the symmetric divisions in the subventricular zone (basal) of the telencephalon (Miyata et al., 2004; Noctor et al., 2004). Accordingly, in the spinal neural tube, it is possible that the early progenitor generates dIL^A and another progenitor in this asymmetric division, then sequentially this late progenitor generates dIL^Bs with a symmetric cell division. Mitosis occurs in the ventricular zone, not the subventricular zone of spinal cord (Altman and Bayer, 1984). Thus, the spatial distinction of two types of progenitors defined by different cell division modes cannot have separable mitotic zones as seen in the telencephalon but may be more like the retina where the nuclei of the progenitors at later stages in a given lineage migrate more basally before the terminal mitosis (Miyata et al., 2004; Noctor et al., 2004; Baye and Link, 2007). Therefore, the spatial segregation of the two $Ascl1^+$ neuronal progenitor populations may be the static representation of heterogenous interkinetic migration in the spinal cord where the dIL^B fate restricted late progenitors have a greater basal nuclei migration. In vivo time lapse imaging to follow single cell lineages will be needed to corroborate this model.

Ascl1 lineage development in gliogenesis

Genetic fate mapping using *Ascl1*^{CreERT2} showed that both astrocytes and oligodendrocytes are derived from $Ascl1^+$ progenitors present at late stages of embryogenesis (E14.5/E15.5). This result is in contrast to the previous fate mapping studies using the BAC Tg line that revealed only oligodendrocyte populations from late Ascl1 lineages (Battiste et al., 2007). In addition, in neurosphere culture assays with cells from rat embryonic spinal cord or mouse neonatal brain showed that Ascl1 is required to generate neurons and oligodendrocytes, but not astrocytes (Parras et al., 2004; Sugimori et al., 2007; Sugimori et al., 2008). However, in the ventricular zone of dorsal spinal cord at

E14.5/E15.5, I identified early Ascl1 expressing progenitors prior to the expression of oligodendrocyte specification markers such as Olig2 or PDGFRα, suggesting this population as the origin of Ascl1 derived astrocytes. This $Ascl1^{+}/Olig2^{-}$ population was missed with the BAC Tg model. Invoking a similar lineage model as for dIL^{A} and dIL^{B} neurons to describe the gliogenesis lineage relationships, early glial progenitors may be bipotential for astrocytes and oligodendrocytes, whereas a later progenitor population is restricted to oligodendrocytes as OPC. The lineages of the common progenitors giving rise to astrocytes and oligodendrocytes such as oligodendrocytes-type-2 astrocyte (O-2A) progenitors have been described using in vitro culture from rat optic nerve or spinal cord (Raff, 1989; Nishiyama et al., 1996). The time lapse imaging to lineage trace the single clones in vivo would corroborate the Ascl1 derived glial lineage model and reveal how this would compare to in vitro models.

Interestingly, Ascl1 appears to be required only for the generation of correct oligodendrocyte numbers. Specific loss of only one cell type in the Ascl1 glial lineage is reminiscent of the specific loss of GABAergic neuron dIL^{A} in neurogenesis. This places the role of Ascl1, possibly opposing Notch signaling, in binary cell choices. In addition, this possibility likely reflects the important role of Ascl1 in setting up asymmetric divisions that allow the generation of distinct cell types from a progenitor.

Only recently has the origin of oligodendrocytes from the dorsal neural tube at later stages of embryogenesis been appreciated (Cai et al., 2005; Fogarty et al., 2005; Vallstedt et al., 2005). These oligodendrocytes are distinct from those that arise from the pMN domain of the early neural tube (~E11) (Lu et al., 2000; Zhou and Anderson, 2002). In addition to differences in the origin of the cells, genetic differences include Ascl1 expression and the requirement for Shh signaling (Fig. 3.9) (Cai et al., 2005; Fogarty et al., 2005). These distinctions raise important questions as to whether this second oligodendrocyte population is

functionally distinct as well. Electron micrographs of oligodendrocytes derived from the Ascl1 lineage clearly showed that at least some can form myelin in the adult nervous system and thus serve as functionally mature oligodendrocytes. Are there any functional features that distinguish these oligodendrocytes? Although genetic tools distinguish these two populations, the assays to probe different aspects of oligodendrocyte function from physiology to animal behavior need to be developed to answer this question.

Cell autonomous functions of Ascl1 in vertebrate neural development

Ascl1 functions in a feedback loop with Notch signaling to regulate neural differentiation in progenitor populations. Because of its obvious role in cell-cell signaling, the analyses of the *Ascl1* null mutant could not distinguish whether the phenotypes observed were due to a mixture of cell autonomous and non-cell autonomous effects. The paradigm using the conditional allele of *Ascl1* with tamoxifen inducible Cre creates a mosaic which allows a cell autonomous function to be more clearly evaluated. In this mosaic analysis, the fates of *Ascl1* mutant cells, in a largely wild type environment, were determined as the spinal cord matured. During neurogenesis, *Ascl1* mutant cells were less likely to become dIL^A neurons and more likely to become dIL^B. Similarly, during gliogenesis the *Ascl1* mutant cells were less likely to become oligodendrocytes, but the astrocyte lineage seems unaffected. The specific requirements of Ascl1 for only one subtype in a given neurogenic or gliogenic lineage emphasizes its role in regulating cell type diversification. Interestingly, the mouse mutant analyses of Notch components such as *Psen1* (presenillin1), *RBPj*, or *Nicastrin* showed opposite phenotypes to that of Ascl1 in neurogenesis and gliogenesis. In $Psen1^{-/-}$ spinal cord where the levels of Notch signaling are reduced, the number of dIL^B neurons decreased, whereas the number of dIL^A neurons was unaffected (Mizuguchi et al., 2006). CNS specific ablation of *RBPj* or *Nicastrin*, two other in

vivo models for disrupting Notch signaling, led to increase oligodendrocyte number, opposite to what have been observed in *Ascl1* conditional mutant (Taylor et al., 2007) (see chapter four). It is also of importance to note that Ascl1 expression remarkably increased in the mouse mutants for *Psen1* or *Nicastrin* (Mizuguchi et al., 2006) (see chapter four). Therefore, it is reasonable to suggest that the activity of Ascl1 in balance with the Notch signaling pathway is essential for the correct choices between two different neuronal or glia subtypes. Supporting the idea that Ascl1 exerts its function as a major player of Notch signaling network, recent studies showed that the Notch ligands *Dll1* and *Dll3* are in vivo downstream targets of Ascl1 (Castro et al., 2006; Henke et al., 2009). Understanding the exact molecular mechanism of Ascl1 controlling cell type diversity will be an important task. Identification of the full repertoire of shared or distinct transcriptional targets of Ascl1 in neurogenesis and gliogenesis will help to understand the pleiotropic roles of Ascl1 in the vertebrate CNS development.

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CHAPTER FOUR

Nicastrin is required for normal gliogenesis in the developing spinal cord

INTRODUCTION

Notch signaling pathway is one of the most fundamental mechanisms regulating cell type diversity in the developing nervous system. Notch signaling and its interplay with proneural bHLH transcription factors controls binary cell fate choices by setting up the unequal distribution of Notch receptors, ligands, and proneural bHLH proteins among otherwise homogenous progenitor cells (Louvi and Artavanis-Tsakonas, 2006) (Fig. 4.1). Using mouse genetics or in vitro culture system, it has been demonstrated that Notch signaling is involved in many different levels of the vertebrate neurodevelopment: neural stem cell maintenance and differentiation, the lineage decision between neurons and glia, and even the fate choices between different neuronal subtypes or between glia subtypes (Yoon and Gaiano, 2005; Louvi and Artavanis-Tsakonas, 2006; Mizuguchi et al., 2006; Peng et al., 2007; Taylor et al., 2007). However, due to the early lethality of most Notch component null embryos before the onset of neurogenesis (E10) and the limitation of in vitro or gain of function studies (Swiatek et al., 1994; de la Pompa et al., 1997; Hamada et al., 1999; Xue et al., 1999; Krebs et al., 2003), the physiological evidence for the importance of Notch signaling on different aspects of neurogenesis and gliogenesis in vertebrates remains limited.

Nicastrin is an indispensable component of Notch signaling as a requisite subunit of the γ-secretase complex, which is essential for activating Notch pathways by cleaving the Notch receptor to generate the Notch intracellular

domain (NICD) (Yu et al., 2000; Chen et al., 2001; Chung and Struhl, 2001; Shah et al., 2005) (Fig. 4.1). Fibroblasts from Nicastrin null embryos fail to release NICD, suggesting Nicastrin can be used to probe the in vivo functions of Notch signaling in the neurodevelopment (Li et al., 2003a). However, Nicastrin null embryos die at E10.5 before the onset of neurogenesis (Li et al., 2003a; Li et al., 2003b), which prevents the opportunity to investigate the roles of Nicastrin and its influence on Notch signaling at the later stages of neurodevelopment.

Here, I investigated in vivo functions of Nicastrin in neurogenesis and gliogenesis of the developing spinal cord, using *Nestin-Cre;Nicastrin*$^{fl/fl}$, the central nervous system (CNS) specific conditional Nicastrin mutant. *Nestin-Cre;Nicastrin*$^{fl/fl}$ embryos had a substantial increase in the number of cells expressing the bHLH factor Ascl1 and a subsequent increase in oligodendrocyte cell number with no defect in neurogenesis detected. The increased oligodendrocyte number is consistent with phenotypes reported from *Nestin-Cre;RBPj*$^{fl/fl}$ embryos, a mouse model for CNS specific deletion of transcriptional Notch effector, RBPj (Taylor et al., 2007). Moreover, this phenotype is opposite to the decreased oligodendrocyte numbers in *Ascl1* mutant spinal cords, consistent with the suggested role for Ascl1 as a counterpart of the Notch signaling pathway (Sugimori et al., 2007, see chapter three). Thus, Nicastrin-containing γ-secretase is essential for Notch signaling and controlling the generation of oligodendrocytes in the spinal cord. These data support the importance of Notch signaling in constraining levels of the bHLH factor Ascl1 which in turn is required in oligodendrogenesis.

MATERIALS AND METHODS

Transgenic mice

Nestin-Cre is the transgenic mouse expressing Cre recombinase under the control of the rat nestin promoter and enhancer (Tronche et al., 1999). The conditional allele of *Nicastrin* (*Nicastrin*^{fl}) has exon 3 of *Nicastrin* flanked by two loxP sites and was described previously (Tabuchi et al., 2009). To obtain CNS specific Nicastrin knockout (*Nestin-Cre;Nicastrin*$^{fl/fl}$) embryos, *Nestin-Cre;Nicastrin*$^{fl/+}$ mice were crossed with *Nicastrin*$^{fl/+}$ mice. The littermates with *Nicastrin*$^{fl/fl}$ or *Nestin-Cre* but not both were used as control. For PCR genotyping, the following primers were used on genomic DNA isolated from yolk sac tissue. For detection of the Cre gene in transgenic embryos: Cre forward, 5'-GGACATGTTCAGGGATCGCCAGGCG-3' and Cre reverse, 5'-GCATAACCAGTGAAACAGCATTGCTG-3' were used and yielded a 300 bp product. For detection of the wild-type or mutant *Nicastrin* allele: Nicastrin forward, 5'-AGCTCTTCACCAGGTAAGAAC-3', Nicastrin reverse, 5'-TTGGACAGTCCTTCCCTGAAG-3', and Nicastrin REC forward, 5'-GGTTCTATCCCACAGTCTGTG-3' were used and yielded a 200 bp PCR product for wild-type, 240 bp for the loxP allele, and 500 bp for the allele after Cre-mediated recombination.

Embryonic tissue preparation and immunofluorescence

Transgenic embryos were harvested at E10.5 or E16.5. The E10.5 embryos were fixed in 4% paraformaldehyde for 2 hours at 4˚C. For the E16.5 embryos, the spinal columns were dissected out immediately, then fixed in 4% paraformaldehyde overnight at 4˚C. After being washed in cold PBS, the tissues were cryoprotected in 30% sucrose in PBS for E10.5 embryos or 30% sucrose in

H_2O for E16.5 overnight. 30 μm thick sections were collected using a Leica cryostat.

Cryosections for embryonic tissues were incubated with the appropriate dilution of primary antibody in PBS/3% normal donkey serum/0.1% Triton X-100, followed by incubation with the appropriate anti-goat secondary antibodies conjugated with Alexa 488, 594, or 647 (Molecular Probes). Mouse monoclonal antibodies used were: NeuN (1:1000, Chemicon, MAB377), S100β (1:2000, Sigma), Ki67 (1:100, Novacastra), Tuj1 (1:1000, BABCO), and Nestin (1:10, Developmental Hybridoma Bank). Rabbit polyclonal antibodies used were: Sox2 (1:3000, Chemicon), Pax2 (1:200, Invitrogen), Tlx3 (1:3000, gift T. Muller), and Olig2 (1:2000, Chemicon). Guinea pig polyclonal antibodies used were: Ascl1 (1:10,000) and Sox10 (1:2000, gift M. Wegner). Confocal imaging was carried out with a Bio-Rad MRC 1024 confocal microscope. For each experiment, multiple sections from at least three animals were analyzed except for the astrocyte phenotype analysis using s100β where only one embryo was used.

RESULTS

No detectable defects in neurogenesis in the spinal cord of *Nestin-Cre;Nicastrin$^{fl/fl}$* embryos

To study the function of Nicastrin in neural development of the spinal cord, *Nestin-Cre;Nicastrin$^{fl/fl}$* mouse embryos were generated to conditionally delete *Nicastrin* from neural stem/progenitors and to bypass the early embryonic lethality of *Nicastrin* null embryos (Tabuchi et al., 2009). As previous studies using *Notch1*, or *Hes1* and *Hes5*, or *RBPj* null embryos have shown that Notch signaling defects lead to premature differentiation of neurons and depletion of neural progenitors (de la Pompa et al., 1997; Ohtsuka et al., 1999), the possible defects in neurogenesis were investigated in the *Nestin-Cre;Nicastrin$^{fl/fl}$* spinal neural tube. At both E10.5 and E16.5 stages, no gross morphological phenotype was observed in *Nestin-Cre;Nicastrin$^{fl/fl}$* embryos and the control littermates. At E10.5, the early neurogenic period, no defects were detected in *Nestin-Cre;Nicastrin$^{fl/fl}$* embryos compared to the controls in the numbers or patterning of Nestin^{+} cells (early neural stem/ progenitor marker), Ki67^{+} cells (proliferation) and Tuj1^{+} cells (differentiation) (Fig. 4.2A-C'). To examine possible neuronal subtype changes in *Nestin-Cre;Nicastrin$^{fl/fl}$* embryos, E16.5 spinal neural tubes were assayed. No significant changes in the total number of neurons (NeuN^{+}) were observed in the *Nestin-Cre;Nicastrin$^{fl/fl}$* embryos compared to controls. In addition, the staining of Pax2, inhibitory neuronal marker, or Tlx3, excitatory neuronal marker, did not show any obvious differences between *Nestin-Cre;Nicastrin$^{fl/fl}$* embryos and the control littermates (Fig. 4.2D-F'). Thus, although Nestin-Cre is predicted to flox out exon 3 of *Nicastrin* in stem/progenitor cells, there was no obvious neurogenesis phenotype was detected.

Nicastrin mutation leads to an increase in oligodendrocyte number in late stage embryonic spinal cord

Notch signaling has been known to regulate gliogenesis in the vertebrate development. Overexpression of activated Notch1, or Hes1 or Hes5 promotes the generation of Müller glia in the mouse or chick retina. A study using CNS specific deletion of *Rbpj* using *Nestin-Cre* showed that RBPj mediated classical Notch signaling inhibits oligodendrogenesis but promotes astrogenesis in the mouse spinal cord (Taylor et al., 2007). To determine if the Nicastrin mutant would phenocopy other notch pathway mutants, I examined the expression of oligodendrocyte and astrocyte lineage markers in E16.5 *Nestin-Cre;Nicastrin*$^{fl/fl}$ spinal cord. These markers include Olig2 and Sox10 which are transcription factors essential for the generation of oligodendrocytes (Lu et al., 2000; Zhou et al., 2001; Stolt et al., 2002). Their expression is initiated at the progenitor stage and sustained in mature oligodendrocytes. GFAP was used as an astrocyte marker as it marks an astrocyte specific intermediate filament protein. And finally, Ascl1 was used as it is a player of Notch signaling pathway and is expressed in glial progenitors in late stages of embryonic spinal cord.

There were significant defects in gliogenesis in E16.5 *Nestin-Cre;Nicastrin*$^{fl/fl}$ embryos, but no detectable defects in neurogenesis. Sox10^{+} or Olig2^{+} oligodendrocyte lineage cells are increased throughout all regions of the spinal cord (Fig. 4.3C-C', G-G'). In addition, the number of cells expressing Ascl1 was also dramatically increased (Fig. 4.3D-D'). The expression of Sox2, a stem cell/progenitor marker, does not change significantly (Fig. 4.3B-B', E-E'). It is of note that the increase of Ascl1^{+} Olig2^{+} cells was more dramatic in the grey matter where the oligodendrocyte precursor cells (OPCs) reside compared to the ventricular zone (Fig. 4.3G-G'). This suggests Nicastrin normally suppresses the generation of oligodendrocyte restricted precursors. At the stage examined, astrogenesis based on GFAP or S100β expression did not seem to be affected

(Fig. 4.3H-H'). However, further examination is necessary using other astrocyte markers at more mature stages of the spinal cord to rule out Nicastrin function in this lineage.

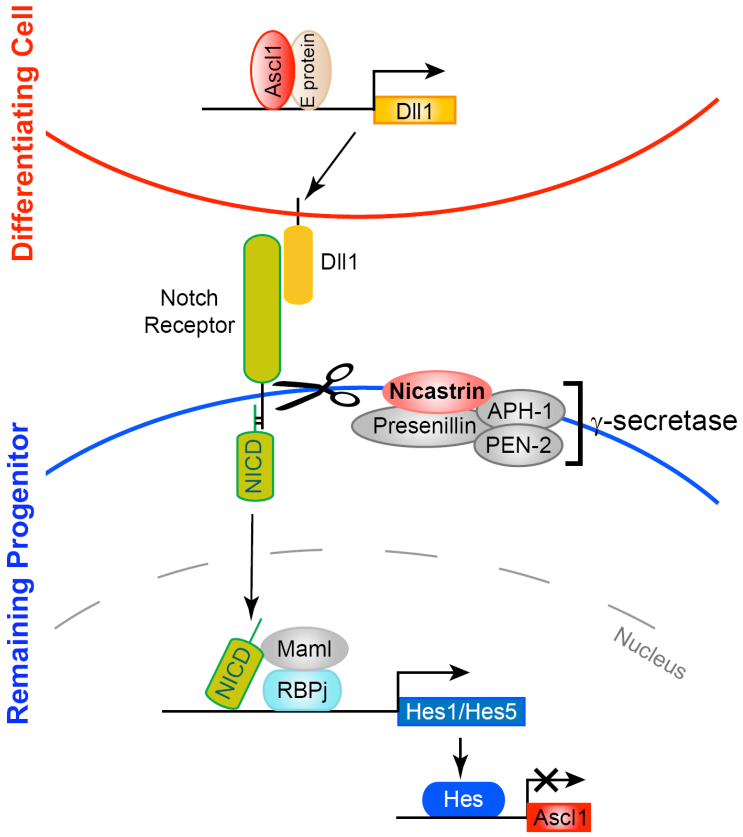

Figure 4.1. Nicastrin control of the Notch signaling pathway as a γ-secretase complex component. In one cell, a proneural basic helix-loop-helix transcription factor such as Ascl1 activates the transcription of Notch ligands such as *Dll1*. Upregulation of Dll1 will activate Notch receptors in neighboring cells. The intracellular domain of activated Notch receptor (NICD) should be cleaved by γ-secretase complex comprising four components (Nicastrin, Presenillin, APH-1, and PEN-2). Translocated NICD forms a transcription activator complex with RBPj and a co-activator Maml (mastermind-like) in the nucleus, upregulating transcription of genes encoding bHLH factors such as *Hes1* or *Hes5*. Hes1 and 5 repress the transcription of proneural genes such as *Ascl1*. The differences in neighboring cells will be amplified. Dll1 and Ascl1 in one cell results in that cell transitioning to a differentiated state, while Hes plus other Notch downstream targets in the other cell keep that cell as a progenitor. Thus, Nicastrin as a requisite component of the γ-secretase complex is involved in controlling Notch mediated binary cell fate decisions. Figure is modified from Kageyama et al., 2008.

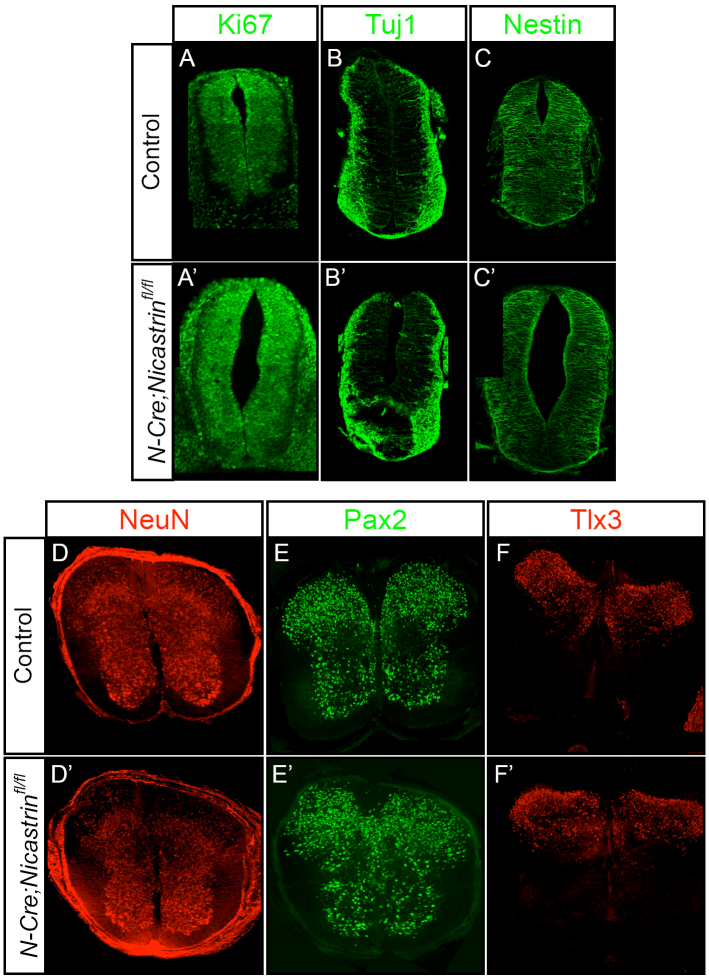

Figure 4.2. No neurogenesis defects detected for *Nestin-Cre;Nicastrin*$^{fl/fl}$ in the developing spinal cord.
(A-C') Early neurogenesis phenotypes such as differentiation or proliferation were examined by immunofluorescence in the spinal neural tubes from E10.5 *Nestin-Cre(N-Cre);Nicastrin*$^{fl/fl}$ (*Nicastrin*^{cKO}) embryos and control littermates. Ki67 (proliferation marker), Tuj1 (differentiation marker), and Nestin (neural stem cell/progenitor markers). (D-F') At E16.5, no obvious neuronal subtype differences were observed between the *Nicastrin*^{cKO} embryos and the control. NeuN (pan-neuronal marker) (D-D'), Pax2 (inhibitory neuronal marker) (E-E'), Tlx3 (excitatory neuronal marker) (F-F'). Abbr: *N-Cre, Nestin-Cre*.

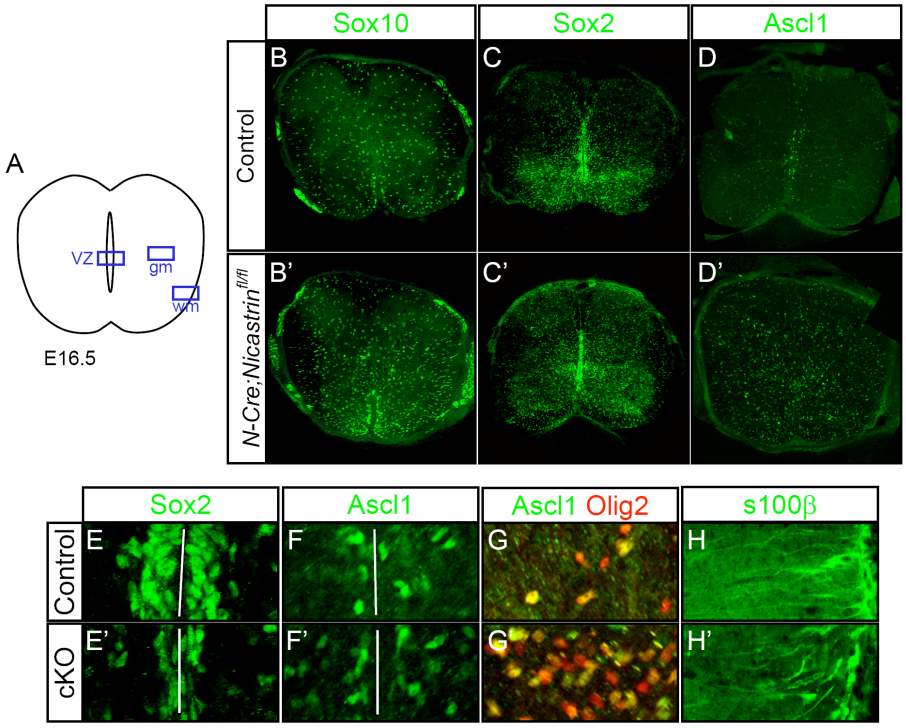

Figure 4.3. Nicastrin inhibits oligodendrocyte formation in the developing spinal cord.

(A) Illustration depicting the transverse section of E16.5 mouse spinal cord (B-B') E16.5 *Nicastrin*^{cKO} (*Nestin-Cre;Nicatrin*$^{fl/fl}$) embryos show an increase in Sox10, an oligodendrocyte lineage marker, compared to the control throughout the spinal cord. (C-C', E-E') Sox2, a marker for progenitor cells appears decreased in the ventricular zone of *Nicastrin*^{cKO} mutants with no obvious change in the number of cells in the gray and white matter (C-C'). (D-D') A dramatic increase of Ascl1 expression is seen in the *Nicastrin*^{cKO} spinal cord. The most obvious increase of Ascl1 is in Olig2^{+} oligodendrocyte precursors in the grey and white matter (G-G'), whereas there is not much change in the ventricular zone (F-F'). There is no obvious change in astrocytes as detected with s100β in E16.5 *Nicastrin*^{cKO} embryos (H-H'). Abbr: *N-Cre*, *Nestin-Cre*; cKO, *Nicastrin*^{cKO}.

DISCUSSION

This is the first study demonstrating an in vivo function for Nicastrin in embryonic neurodevelopment. CNS specific deletion of *Nicastrin* leads to an increased number of oligodendrocyte progenitors expressing Sox10, Olig2, and Ascl1 in the developing spinal cord. Since Nicastrin functions as a component of the γ-secretase complex that is required for processing the Notch receptor, the gliogenesis phenotype supports the idea that Notch has an inhibitory role in oligodendrogenesis. This was concluded from studies using activated Notch1, Hes1, or Hes5 that suggested Notch signaling inhibits oligodendrocyte differentiation in rat optic nerve, adult hippocampal cell cultures or in the zebrafish spinal cord (Wang et al., 1998; Tanigaki et al., 2001; Park and Appel, 2003). More recently, CNS specific ablation of *RBPj*, the downstream transcriptional effector for Notch signaling, also shows an increased number of oligodendrocytes at the expense of astrocytes in the mouse spinal cord (Taylor et al., 2007).

One consideration to keep in mind in interpreting *Nestin-Cre;Nicastrin*$^{fl/fl}$ analysis is that Nicastrin-containing γ-secretase can cleave not only Notch receptors but also other single pass transmembrane proteins such as amyloid precursor protein (APP) or ErbB4 (Fortini, 2002; Selkoe and Kopan, 2003). Therefore, I cannot exclude the possibility that the defects reported here for Nicastrin conditional knockout are due to other γ-secretase substrates. Although ErbB4 mediated signaling is known to mediate neural precursor differentiation, the generation of astrocytes is increased in the telencephalon of Erb4 knockout embryos, which is not in accordance with the phenotypes of the Nicastrin mutant or other studies using Notch components (Sardi et al., 2006). APP is expressed mainly in the differentiated neurons (Citron et al., 1992; De Strooper and Annaert, 2000). Although the biological function of APP is not well understood, it

has been implicated as a regulator of synapse formation (Priller et al., 2006). Taken together, I would argue that the phenotype of conditional *Nicastrin* mutants is likely through defects in Notch signaling. At least two future experiments would help clarify the contribution of these different γ-secretase substrates to the phenotype. First, the levels of the processed forms of each γ-secretase substrate in the *Nicastrin* mutant should be assessed. Second, functional studies such as rescue experiments using mouse genetics or pharmacology will reveal which signaling pathway is the major contributor for the gliogenesis phenotypes.

What would be the molecular mechanism of Nicastrin involvement in gliogenesis? It is known that in the absence of Nicastrin there is no NICD, and thus, no active Notch signaling (Li et al., 2003a; Li et al., 2003b). Therefore, the suppression of proneural bHLH transcription such as Ascl1 by activated Notch will be disinhibited. The upregulated Ascl1 activity in *Nicastrin* mutants would promote oligodendrogenesis, consistent with its known functions. Together with the increased level of Ascl1, the investigation of the changes in other Notch components in *Nestin-Cre;Nicastrin*$^{fl/fl}$ such as NICD or Hes1/5 would help to understand the relation or interaction between Nicastrin and Notch signaling pathway in gliogenesis.

It remains to be addressed whether the maturation of the supernummery oligodendrocyte progenitor cells is also affected in the Nicastrin mutant. A role for Notch signaling in later stages of oligodendrocyte development has been suggested (Louvi and Artavanis-Tsakonas, 2006; Taylor et al., 2007). Expression of Jagged1, a Notch ligand, inhibits oligodendrocyte maturation and myelination (Wang et al., 1998). In *Notch1* heterozygous mice, premature myelination was observed (Givogri et al., 2002). Examine spinal cords from these mutant mice at later stages with different mature oligodendrocyte markers such as MBP (myelin basic protein) or CNPase would help to answer this question. Another question that could be answered by examining later stages of spinal cord development is

whether an astrocyte phenotype becomes evident. The lack of an astrocyte phenotype at E16.5 does not preclude a disruption in this lineage. In the developing retina and telencephalon and in cell culture systems, overexpression of Hes1 or Hes5 promotes the differentiation of astrocytes. Moreover, *Nestin-Cre;Rbpj*$^{fl/fl}$, another in vivo mouse model for disrupting Notch signaling, clearly showed a reduction of astrocyte number in the neural tube (Taylor et al., 2007). This discrepancy is possibly due to the differences in the embryonic stages used to analyze the phenotype. Whereas E16.5 spinal cord was examined in this study, E19.5 spinal cord was assessed in the study of *RBPj* conditional mutants. Thus, it is necessary to examine later stage *Nestin-Cre;Nicastrin*$^{fl/fl}$ spinal cords, as astrocyte lineage markers such as GFAP or glutamine synthetase express more clearly in the mature spinal cord.

In contrast to numerous previous studies demonstrating Notch function in neurogenesis, no significant phenotypes for neurogenesis were observed in the conditional *Nicastrin* mutants examined here. It is possible that Nicastrin is not required for Notch signaling during neurogenesis but this is unlikely as no other Nicastrin related protein has been identified as a component in the γ-secretase complex. A more likely explanation is that Nicastrin has a long half-life (Herreman et al., 2003), longer than other components of the Notch pathway previously tested. Since Nestin-Cre mediated *Nicastrin* deletion would start presumably around E9.5-10, it is possible that Nicastrin proteins generated earlier than E9 still remain to function during the neurogenic period (E10-11). Detection of Nicastrin protein and NICD in *Nestin-Cre;Nicastrin*$^{f/f}$ neural tube at this early embryonic stage would help to investigate this possibility.

Although it still remains as an important task to delineate the clear relationship between Nicastrin, Ascl1, and Notch pathway for cell type diversification, this study suggests the conditional mutant of Nicastrin as a

valuable model to elucidate the physiological role of Notch signaling in later stages of embryogenesis.

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CHAPTER FIVE

In vivo analysis of Ascl1 defined progenitors reveals distinct developmental dynamics during adult neurogenesis and gliogenesis

INTRODUCTION

Although most neural cell differentiation and maturation events forming the adult nervous system are completed by early postnatal periods, mitotic precursor cells for neurons, oligodendrocytes, and astrocytes continue to exist throughout the adult brain. The glial precursors such as reactive astrocytes and oligodendrocyte precursor cells (OPCs) are spread broadly throughout the adult brain (Ridet et al., 1997; Polito and Reynolds, 2005). Astrocytes are generated in response to brain injury and OPCs are slowly maturing cells that respond rapidly to demyelinating conditions (Gensert and Goldman, 1997; Ridet et al., 1997; Chari and Blakemore, 2002). In contrast, adult generated neurons arise from two restricted regions; the subgranular zone (SGZ) in the dentate gyrus of the hippocampus, and the subventricular zone (SVZ) of the lateral ventricles generating olfactory bulb interneurons via the rostral migratory stream (RMS) (Gage, 2000; Alvarez-Buylla and Lim, 2004). The molecular control of differentiation of these adult generated neural cell-types is much less studied than that for neural differentiation during development. However, many of the important regulatory control genes functioning during embryonic neural development are present in neuronal and glial precursors in the adult, providing models of how adult generation of neural

cell-types might occur. Here I define precursors in adult brain expressing one such differentiation control factor, Ascl1 (previously Mash1), and I follow the progression of these cells as they differentiate to neurons and oligodendrocytes.

Ascl1 is present transiently in neural progenitor cells in complex temporal and spatial patterns throughout neural development. A mouse mutation null for *Ascl1* has been studied extensively and has defined Ascl1 as a neuronal differentiation control factor in multiple regions of the developing central and peripheral nervous systems (reviewed by Bertrand et al., 2002). In paradigms where Ascl1 is ectopically expressed in neural progenitor cells, the cells exit the cell-cycle and begin expressing neuronal differentiation markers (Farah et al., 2000; Nakada et al., 2004). However, within a particular spatial and temporal context, Ascl1 appears to play a role in the specification of neuronal sub-type as seen in interneuron formation in the spinal cord (Nakada et al., 2004; Helms et al., 2005) and cortex (Fode et al., 2000; Parras et al., 2002), and in sympathetic neurons during neural crest development (Perez et al., 1999). Thus, Ascl1 is an important regulator of neuronal differentiation and sub-type specification in the developing central and peripheral nervous systems.

In addition to its role in neurogenesis, Ascl1 also plays a role in oligodendrocyte development. Ascl1 expression has been reported in OPCs in culture (Kondo and Raff, 2000; Wang et al., 2001; Gokhan et al., 2005), and forced expression of Ascl1 in neural progenitor cultures biases fate to neurons and oligodendrocytes but not astrocytes (Gokhan et al., 2005; Sugimori et al., 2007). Lineage tracing of Ascl1 expressing cells in spinal neural tube development revealed these cells are fated to neurons and oligodendrocytes but not astrocytes in vivo (Battiste et al., 2007). At postnatal day 0 (P0) Ascl1 cells in the SVZ/RMS also have an oligodendrocyte producing component (Parras et al., 2004). A reduced number of oligodendrocytes were reported in the telencephalon of Ascl1 mutant embryos, suggesting Ascl1 functions in the oligodendrocyte lineage to

generate the normal numbers of cells (Parras et al., 2007). Furthermore, subsets of neuroblastomas and oligodendrogliomas express Ascl1 consistent with restriction of Ascl1 to precursors of these two neural fates that may reside in the adult brain (Rousseau et al., 2006). Together these studies place Ascl1 in important regulatory roles controlling neuronal and oligodendrocyte differentiation during embryogenesis, and make Ascl1 a strong candidate for playing a role in these processes in the adult brain.

Here I use a combination of genetically modified mice to definitively place Ascl1 in adult brain in progenitor cells that are transitioning to mature neurons or oligodendrocytes. Ascl1 transiently identifies a variety of neural progenitor cells early in each lineage including Type-1 stem like cells and Type-2a cells in the dentate gyrus of the hippocampus, transit amplifying cells in the SVZ and RMS in the forebrain, and immature OPCs dispersed widely in subcortical gray matter and in a ventral strip in the white matter tract of the corpus callosum. Cells that express high levels of Ascl1 transition to more mature phenotypes; they do not remain as progenitors but progress to differentiated cell-types at different maturation rates in each lineage. These characteristics of Ascl1 in the adult CNS lineages are analogous to the temporal placement of Ascl1 during embryonic development revealing molecular commonality in these temporally distinct processes.

MATERIALS AND METHODS

Transgenic mice and Tamoxifen treatment

Ascl1-CreER^{TM}, *Ascl1^{CreERT2}*, *R26R-stop-YFP* and *Ascl1^{GFP}* mice were described previously. Briefly, *Ascl1-CreER^{TM}* is a BAC transgenic mouse where CreER^{TM} replaces the Ascl1 coding sequence (Battiste et al., 2007). *Ascl1^{CreERT2}* is an inducible Cre knock-in strain where CreERT2 is inserted in the locus of Ascl1 (see Chapter three). *R26R-stop-YFP* is a Cre recombinase reporter strain (Srinivas et al., 2001). *Ascl1^{GFP}* has GFP knocked into the Ascl1 locus replacing the Ascl1 coding sequence and otherwise only modifying the locus with a short linker sequence around the ATG (Leung et al., 2007). Adult mice (postnatal day 60, P60) with the genotype *Ascl1-CreER^{TM}/+;R26R-stop-YFP/+* were injected intraperitoneally with 300 mg tamoxifen (Sigma) in sunflower seed oil per kg of body weight on two consecutive days. P45 or P60 *Ascl1$^{CreERT2/+}$;R26R-stop-YFP/+* mice were injected with 180 mg tamoxifen per kg of body weight on five consecutive days. Brains were collected 5, 7, 30, or 180 days after tamoxifen treatment following Avertin anesthesia and trans-cardiac perfusion with 4% paraformaldehyde. Brains were fixed further by immersion in 4% paraformaldehyde overnight at 4 ^{o}C, rinsed in PBS, cryoprotected in 30% sucrose, embedded and frozen in OCT, and 40-50 μm cryosections generated. P60 *Ascl1^{GFP}* mice were perfused and brains processed as above.

For Cre induction at embryonic stages, pregnant dams carrying E11.5 *Ascl1$^{CreERT2/+}$;R26R-stop-YFP/+* embryos were tamoxifen (50-75 mg/kg) administered interperitoneally. The embryonic brains were harvested and prepared for immunofluorescence as described in Chapter two and three.

Immunofluorescence and BrdU labeling

Free floating sections were incubated with the appropriate dilution of primary antibody in PBS/0.5% normal donkey serum/0.2% NP-40, followed by incubation

with the appropriate anti-goat or anti-donkey secondary antibodies conjugated with Alexa 488, 594, or 647 (Molecular Probes). Mouse monoclonal antibodies used were: GFAP (1:400, Sigma-Aldrich, G3893), BrdU (1:25, BD Biosciences, 347580), Ascl1 (1:25, Pharmingen), Nestin (1:2000; BD Pharmingen), NeuN (1:1000, Chemicon, MAB377), Ki67 (1:100, Novacastra), GAD67 (1:1000, Chemicon), Calretinin (1:500, Chemicon), Glutamine synthetase (1:500, Chemicon), PDGFRα (1:200, BD Biosciences), and APC (1:100, Oncogene Sciences, clone CC-1). Rabbit polyclonal antibodies used were: GFP (1:500, Molecular Probes, A6455), GFAP (1:500, DAKO), Sox2 (1:3000, Chemicon), BLBP (1:20, a gift from G. Fishell), Calbindin (1:1000, Swant), TH (1:2500, Chemicon), Dlx (1:75, gift J. Kohtz), and Olig2 (1:2000, Chemicon). Goat polyclonal antibodies used were: anti-NeuroD (1:200, Santa Cruz) and anti-Doublecortin (Dcx) (1:200, Santa Cruz). Chick anti-GFP (1:500, Aves lab) and guinea pig anti-Sox10 (1:2000, gift M. Wegner) were also used. For BrdU labeling, animals were injected intraperitoneally once with BrdU at 150 mg/kg 2 hours before sacrifice. Confocal imaging was carried out with a Bio-Rad MRC 1024 or Zeiss LSM510 confocal microscope. For each experiment, multiple sections from at least 3 animals were analyzed except for the 6 month timepoint where 2 animals were analyzed.

RESULTS

Characterization of Ascl1 in the adult brain

Several reports have placed Ascl1 in dentate gyrus of hippocampus and the SVZ of the lateral ventricles in the adult brain (Pleasure et al., 2000; Kohwi et al., 2005; Kuo et al., 2006). I confirmed these reports (Fig. 5.1H) and set out to obtain a more precise description of Ascl1 expression in the adult brain. Given the low sensitivity of Ascl1 antibodies in the adult brain, I turned to a mouse model where GFP was knocked into the Ascl1 locus (*Ascl1*^{GFP}) to perform this analysis ((Leung et al., 2007) and Fig. 5.1A). The use of GFP in the knockin mouse to indicate Ascl1 expression has been validated in the olfactory epithelium (Leung et al., 2007) and here in E11.5 neural tubes (Fig. 5.1B,C) and adult dentate gyrus (Fig. 5.1H-H"). As previously reported, Ascl1 is spatially restricted in the ventricular zone along the rostral-caudal and dorsal-ventral axis in the developing neural tube, and disappears in more lateral regions as the cells exit the cell cycle and begin to differentiate. In *Ascl1*$^{GFP/+}$ embryos, GFP and Ascl1 are co-expressed within the ventricular zone. However, since GFP protein is more stable than Ascl1, it persists laterally into more differentiated cells (Fig. 5.1B,C). Thus, *Ascl1*$^{GFP/+}$ can be used to identify cells that express or have recently expressed Ascl1.

Analysis of P60 brains from *Ascl1*$^{GFP/+}$ mice revealed many GFP cells scattered throughout multiple regions (Fig. 5.1D). GFP was notable in the two adult neurogenic zones, the SGZ in the hippocampus and the SVZ around the lateral ventricle (Fig. 5.1E,F,H'). GFP cells were also abundant in white matter including the corpus callosum, fimbria, and anterior commissure (Fig. 5.1D, and data not shown). Even in gray matter such as the thalamus, scattered GFP cells were detected although expression was weaker than in other regions (Fig. 5.1G). Selective expression of GFP in the neurogenic zones in the hippocampus and

lateral ventricles, but not in mature neuronal cell layers in the dentate gyrus or olfactory bulb suggests Ascl1 is transiently expressed during adult neurogenesis. In addition, the extensive, dispersed GFP expression throughout the adult brain including white matter tracts is consistent with recent reports that place Ascl1 in oligodendrocyte precursors (Battiste et al., 2007; Parras et al., 2007; Sugimori et al., 2007).

Ascl1-GFP cells are largely type-2a cells along the adult hippocampal neurogenic lineage and are restricted to the granule neuron cell fate

In the adult hippocampus, putative multipotent neural stem cells reside in the SGZ and have a limited self-renewal capacity (Seri et al., 2001; Seri et al., 2004; Bull and Bartlett, 2005). According to a current model of the adult hippocampal neuronal lineage (for diagram see Fig. 5.2F), a combinatorial set of molecules identifies each stage (Kempermann et al., 2004; Steiner et al., 2006). Briefly, Type-1 neural stem cells have astrocytic features and are marked by GFAP. Although these cells have proliferative capacity, they are much more slowly cycling than the Type-2 progenitor cells. Nestin, Sox2 and BLBP are also expressed in Type-1 cells but they persist into the Type-2 cell stages (Steiner et al., 2006). NeuroD and Doublecortin (Dcx) appear in Type-2b, the later stage of Type-2 cells, and persist into post-mitotic but immature granule cell precursors. Finally, cells mature into NeuN/Calbindin granule cell neurons in the dentate gyrus.

To identify where Ascl1 is relative to this staging scheme, I used co-labeling of GFP from *Ascl1*$^{GFP/+}$ brains with these different markers. The majority of GFP cells co-label with BLBP and Sox2 (Fig. 5.2B,C). By contrast, only few GFP cells can definitively be scored as GFAP^{+}, using the criteria that the GFP cell must be wrapped by GFAP to score it as positive for Type-1 identity (Fig. 5.2A). Since 97% of the GFP^{+} cells co-label with Sox2, they are largely classified as

Type-2a cells. A minority of GFP cells co-express NeuroD (13%) and Dcx, markers of late Type-2b/3 identity (Fig. 5.2D-E). Note that since GFP is more stable than endogenous Ascl1, the overlap with the Type-2b markers is likely an overestimate for the endogenous protein. Furthermore, no GFP cells co-labeled with the mature neuronal marker NeuN (Fig. 5.2B,D,E). These findings place Ascl1 temporally in Type-2a cells in the dentate gyrus.

Although GFP in the *Ascl1*^{GFP} mouse faithfully represents endogenous Ascl1 and the short term fate of these cells, it does not allow us to determine the ultimate fate of the Ascl1 progenitors. In fact, it takes 3-4 weeks for an adult generated neuron to reach a mature neuronal phenotype (van Praag et al., 2002). To map the fate of the Ascl1 lineage cells in the adult brain, I utilized a BAC transgenic mouse line, *Ascl1-CreER*^{TM} that expresses tamoxifen inducible Cre recombinase under the control of *Ascl1* regulatory elements (Battiste et al., 2007) crossed with the Cre reporter line *R26R-stop-YFP* (Srinivas et al., 2001). In the absence of tamoxifen, brains of these animals have no detectable YFP expression. To label the Ascl1 lineage in the adult brain, *Ascl1-CreER*^{TM}*;R26R-stop-YFP* mice were administered tamoxifen at P60 and brains were harvested after 5 days, 30 days or 180 days (Fig. 5.3A). Recombination efficiency of the *Ascl1-CreER*^{TM} line in the adult hippocampus is estimated to be 6% based on comparing the number of GFP and YFP positive cells five days after tamoxifen in the SGZ of *Ascl1*$^{GFP/+}$ and *Ascl1-CreER*^{TM}*;R26R-stop-YFP* mice. Five days after tamoxifen administration, the YFP cells in the dentate gyrus have transitioned from Type-2a to a Type-3/immature neuron stage (Fig. 5.3B-E). This is illustrated by the decrease in the proportion of YFP labeled cells co-expressing Sox2 and the increase in co-expression of NeuroD and Dcx relative to that seen with the Ascl1-GFP. The YFP cells form clusters in the SGZ, a characteristic of immature neuronal precursors (Seri et al., 2004), and they do not exhibit structural characteristics of mature neurons such as branched dendrites and axons (Fig.

5.3B). 10% of the YFP cells incorporated BrdU after 2 hours exposure (n=210 counted, Fig. 5.3C), and 14% co-label with Sox2 (n=88 counted). The majority of YFP cells co-label with NeuroD (55%) and Dcx (Fig. 5.3D,E). No overlap with the differentiated neuronal marker NeuN is seen at this timepoint (Fig. 5.3E). This combination of markers indicates that as early as 5 days after Ascl1 expression, as detected by Cre recombinase activity, the lineage marked cells have largely transitioned from Type2a to a Type3/immature neuron stage.

By thirty days after tamoxifen administration, the majority of YFP cells (86%) have continued to mature and now co-express NeuN and exhibit dendritic processes, characteristics of granule cell neurons (Fig. 5.3F,G). At this timepoint, 14% of the YFP cells in the dentate gyrus retain the morphology of immature progenitors, and a subset co-label with GFAP (Fig. 5.3H). However, by six months YFP cells in the dentate gyrus are almost exclusively in NeuN cells (98%), and they co-express Calbindin a marker of mature granule neurons (Fig. 5.3J,K). The labeled cells continue to elaborate complex dendritic processes (Fig. 5.3I,J). Notably, from the cellular morphology of the YFP cells within the granule zone (Cameron and McKay, 2001; van Praag et al., 2002; Liu et al., 2003), no GABAergic neurons appear to arise from Ascl1 cells in adult dentate gyrus. Taken together, these results demonstrate that the Ascl1 cells detected in this paradigm are largely Type2a progenitor cells that mature into granule cell neurons by 30 days, with a subset marking a stem cell population of limited renewal potential.

Ascl1-GFP cells are transit amplifying cells in the SVZ and neuroblasts along the RMS and are fated to become interneurons in the olfactory bulb

In adult brains of the *Ascl1*$^{GFP/+}$ mice, many GFP cells were detected in the SVZ of the lateral ventricles and along the RMS, placing Ascl1 in this neurogenic niche as well (Fig. 5.1). In the SVZ and RMS, Ascl1 cells express

GFP, but not all GFP cells express Ascl1 (Fig. 5.4A,D). The GFP^{+}/$Ascl1^{-}$ cells likely represent differences in our ability to detect these proteins combined with the stability difference between GFP and Ascl1 previously noted. Using GFP in the *Ascl1*^{GFP} knockin to characterize the temporal and spatial characteristics of *Ascl1* expression in the SVZ and RMS, I co-labeled the GFP cells with a series of markers that identify cells in different stages in the development of this neurogenic region. Ki67 and Sox2 mark proliferating neural progenitor cells. All Ki67 cells and a majority of the Sox2 cells in the SVZ and RMS co-label with GFP, but with no clear co-labeling of the stem cell marker GFAP (Fig. 5.4B,C). This combination of markers is consistent with the majority of Ascl1-GFP cells being the rapidly dividing transit amplifying cells rather than the slowly dividing neural stem cells. In addition, a majority of GFP cells located further along the RMS co-express Dlx and Dcx, identifying them as neuroblasts (Fig. 5.4G,H). A few GFP cells co-label with Olig2 (Fig. 5.4F) consistent with previous reports for Olig2 expression in the RMS (Hack et al., 2005). Notably, the GFP cells extend from the SVZ and into the RMS but are not found in the olfactory bulb and do not co-label with NeuN (Fig. 5.4E). Thus, in the adult brain, Ascl1-GFP cells comprise transit amplifying cell populations and neuroblasts in this neurogenic lineage (Fig. 5.4I).

I utilized the *Ascl1-CreER*^{TM}*;R26R-stop-YFP* mouse to determine the fate of the Ascl1 cells and to further examine if SVZ stem cells express Ascl1. Just five days after tamoxifen administration in a P60 mouse, although some YFP cells were found around the SVZ (Fig. 5.5A), the majority of Ascl1-lineage YFP cells were found along the RMS (Fig. 5.5B,C). As predicted from their location, the YFP cells in the SVZ co-expressed Sox2 (87%) (Fig. 5.5A, inset) and Dcx (93%). In contrast, essentially all the YFP cells in RMS were Dcx neuroblasts (Fig. 5.5D). At this timepoint, YFP cells did not co-label with NeuN and cells had not reached the olfactory bulb (data not shown). In contrast, after thirty days, all YFP

cells were found in the olfactory bulb (Fig. 5.5A'-C'). Strikingly, no YFP cells were found in the SVZ or the RMS (Fig. 5.5A',B'). These results clearly demonstrate that cells expressing Ascl1 at levels detected in this paradigm are not stem cells but rather are transit amplifying cells that within 30 days have completed their migration to the olfactory bulb and differentiated into neurons.

Unlike adult generated granule neurons in hippocampus, which appear to be a uniform neuronal sub-type, newly generated olfactory bulb neurons are heterogeneous in two distinctive layers, the granule cell layer (GCL) and the glomerular layer (GL) (Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994). The GCL and the GL comprises GABAergic neurons, however, in the GCL this appears as a homogeneous population whereas in the GL the cells can be classified as dopaminergic, Calbindin^{+}, or Calretinin^{+} (Kosaka et al., 1995; Kosaka et al., 1998; Saghatelyan et al., 2004; Kohwi et al., 2007). To determine if the Ascl1 lineage contributes to all neuronal sub-types in the olfactory bulb the YFP cells 30 days after tamoxifen treatment were examined with the different markers. Based on the location in the olfactory bulb, 88% of YFP cells were located in the GCL and 12% were found in the GL (780 cells counted). The identity of the YFP cells in the GCL as GABAergic neurons was confirmed by co-labeling with NeuN and the GABAergic marker GAD67 (Fig. 5.5E,F). The YFP cells located in the GL were co-labeled with Calbindin, Calretinin, or the dopaminergic marker TH (Fig. 5.5G-I), indicating the Ascl1-lineage in the GL neurons was not biased towards one specific neuronal sub-type. Thus, other than GABAergic identity, Ascl1 does not appear to specify neuronal sub-type in the olfactory bulb.

The *Ascl1*^{CreERT2} knock-in mice reveal the existence of Ascl1^{+} neural stem cells in the SVZ and the SGZ of adult hippocampus

Whereas the majority of Ascl1^{+} adult progenitors are Type-2a cells in the hippocampus or transit amplifying cells in the SVZ, some Ascl1-GFP cells in P60 *Ascl1*$^{GFP/+}$ brain show the characteristics of adult neural stem cells with GFAP, Type-1 or neural stem cell marker (Fig. 5.2A, data not shown). Although the BAC Tg *Ascl1-CreER*^{TM} is a valuable model to investigate the dynamics of Ascl1^{+} Type-2a or transit amplifying cells during adult neurogenesis, this transgenic line is not efficient in labeling the putative Ascl1^{+} neural stem cells. To characterize the dynamics of the Ascl1 population of adult neural stem cells, I utilized the *Ascl1*$^{CreERT2/+}$ knock-in line where CreER^{T2} was knocked into the endogenous Ascl1 locus (see Chapter three).

To compare the *Ascl1* lineages labeled by *Ascl1*$^{CreERT2/+}$ with those labeled by BAC Tg *Ascl1-CreER*^{TM}, 180 mg/kg/day tamoxifen was administered to P45 or P60 adult *Ascl1*$^{CreERT2/+}$*;R26R-stop-YFP* mice for 5 consecutive days. When the brains were harvested 7 days later, many YFP^{+} cells co-expressing Sox2 or Dcx were found in the SVZ or along the RMS where the stem cell/progenitors for olfactory bulb interneurons reside, whereas none were co-expressing NeuN (Fig. 5.6A-C, data not shown). As expected, 30 days after tamoxifen administration, many YFP^{+} cells co-label with NeuN in the olfactory bulb demonstrating the cells are transitioning to mature neurons (Fig. 5.6.A, G-G'). Notably, in contrast to the fate mapping with the BAC Tg *Ascl1-CreER*^{TM} where essentially all of the YFP cells differentiated to mature granule neurons within 30 days (Fig. 5.5), in the *Ascl1*$^{CreERT2/+}$*;R26R-stop-YFP* brains many YFP cells still remain in the SVZ and RMS 30 days after initial *Ascl1* expression (Fig. 5.6A'-C'). These results suggest the *Ascl1*$^{CreERT2/+}$ line is labeling cells at an earlier stem-like progenitor stage than that seen previously. In addition, a subpopulation of YFP^{+} lineage cells in the SVZ was co-labeled with GFAP (Fig. 5.6F-F'), supporting their identities as self-renewable stem cells.

In the dentate gyrus of the hippocampus of *Ascl1*$^{CreERT2/+}$*;R26R-stop-YFP* mice 7 days after tamoxifen injection, 44% of YFP cells were Sox2^{+} early progenitors with a small proportion (6%) having Type-1 cell morphology (stem cell-like radial glia morphology) (Seri et al., 2001) (Fig. 5.6H-J',N) , and 58% of YFP cells were NeuroD1^{+} Type-2b or 3/immature neurons (Fig. 5.6K-K',N). 30 days after tamoxifen administration, 34% of the YFP cells matured into NeuN^{+} granule neurons, whereas 60% of the cells remained as early or immediate progenitor cell populations (32% Sox2^{+}; 27% NeuroD1^{+}) (Fig. 5.6H'-N). 12% of the YFP cells clearly showed Type-1 cell morphology and their stem cell identity was confirmed with GFAP (Fig. 5.6L-L', N). In addition to identifying Ascl1-GFP^{+}/GFAP^{+} cells in P60 *Ascl1*$^{GFP/+}$ brains (Fig. 5.2A), these fate mapping results using *Ascl1*$^{CreERT2/+}$ mice clearly demonstrates that Ascl1 is expressed in at least some of the self-renewable neural stem cell population in adult neurogenic niches. Indeed, this fate map of *Ascl1*^{CreERT2} is comparable with the fate map of *Nestin-CreER*^{T2} which was shown to mark the Type-1 cells stem-like cells in the adult brain (Lagace et al., 2007).

Ascl1 expressing embryonic neural stem cells give rise to adult neural progenitors in the SVZ

The embryonic origin of adult neural stem cells and their genetic profile are not completely understood. It has been suggested that a small subset of cells in the neuroepithelial germinal zone persists in the SVZ supplying the adult brain stem cell population (Corbin et al., 2008). Several previous studies using transplant experiments or cre-flox genetic fate mapping showed that the adult neural stem cells in the SVZ are mostly derived from progenitor populations in the embryonic ganglion eminence, the germinal zone of ventral telencephalon for GABAergic interneurons (Wichterle et al., 1999; Kohwi et al., 2007; Young et al., 2007; Corbin et al., 2008). These embryonic progenitor pools in the medial (MGE) or

lateral (LGE) compartment are labeled with Nkx2.1 or Gsh2 respectively (Young et al., 2007). However, a cre-flox fate mapping method without inducible control, as have been used for Nkx2.1-Cre or Gsh2-Cre, cannot be used to determine the embryonic origin of the adult neural stem cell population since Nkx2.1 and Gsh2 expressing cells are generated or maintained throughout development (Young et al., 2007). In addition, transplant experiments were done with donor tissue from E14 embryos, a stage midway through neurogenesis (E14-16) (Wichterle et al., 1999). To delve into the genetic history of adult neural stem cells, I utilized the tamoxifen inducible *Ascl1*$^{CreERT2/+}$ knock-in line to determine if Ascl1 expressing embryonic progenitor pools develop into adult neural stem cells in the SVZ.

Ascl1 is expressed in the ganglion eminence, the region suggested as the embryonic origin for the adult neural stem cell niche. Harvesting *Ascl1*$^{GFP/+}$ embryos at E12.5 when early neurogenesis occurs in the telencephalon, most, if not all, Ascl1 or Ascl1-GFP expressing cells in the ganglion eminence were co-labeled with Sox2 (Fig. 5.7A-C''). Sox2 is a stem cell/progenitor marker in the ventricular zone where radial glia like stem cells reside and in the subventricular zone where basal progenitors are located (Doetsch et al., 1999). To determine if any of these Ascl1/Sox2 expressing progenitors have the characteristics of stem cells, tamoxifen was administered at E11.5 to *Ascl1*$^{CreERT2/+}$*;R26R-stop-YFP* embryos. Harvesting these embryonic brains at E14.5, some YFP^{+} Ascl1 lineage cells still remain in the ventricular zone three days after their initial *Ascl1* expression and show radial glia like end feet structures (Fig. 5.8A-B). Harvesting the brains at E17.5, Ascl1 lineage cells are still detected in the VZ of the forebrain (Fig. 5.8C). Interestingly, many Ascl1 lineage cells are clustered, suggesting they are clonally related. In fact, one or two YFP^{+} cells in the clusters have processes reaching to the pia matter, suggesting radial glia like end feet structures whereas other YFP^{+} cells seemed to migrate along the process (Fig. 5.8C). These characteristics suggest Ascl1 defined radial glia like stem cells possibly divide

asymmetrically to generate the clones. Co-expression of BLBP, radia glia marker, along with other progenitor markers such as Sox2 and Olig2 support this interpretation (Fig. 5.8 inset). These cells were fate mapped into the adult by harvesting P30 *Ascl1*$^{CreERT2/+}$*/R26R-stop-YFP* brains after E11.5 tamoxifen administration (Fig. 5.8D-E''). A few YFP^{+} *Ascl1* lineage cells reside in both dorsal and ventral regions of the SVZ, the adult neurogenic niche (Fig. 5.8D-E). Their molecular profiles, Sox2^{+}/NeuN^{-}, and their immature progenitor like morphology identify them as adult neural progenitors (Fig. 5.8D'-D'', E'-E''). These findings suggest at least some adult neural stem-like/progenitor cells are derived from embryonic neural stem cells which had expressed Ascl1 during early neurogenesis of the telencephalon.

Ascl1 is an early marker for adult oligodendrocyte progenitor cells

Oligodendrocytes are generated during embryogenesis and throughout adult life. Adult oligodendrocyte progenitor cells (OPCs) are identified by PDGFRα or NG2 and constitute the major dividing cell population contributing to myelination repair in response to brain injury (Chari and Blakemore, 2002; Reynolds et al., 2002; Dawson et al., 2003; Woodruff et al., 2004; Polito and Reynolds, 2005). Ascl1 is present in oligodendrocyte progenitors during embryogenesis, in early postnatal brain, and in the adult brain (Parras et al., 2004; Battiste et al., 2007; Parras et al., 2007). I investigated Ascl1 in this lineage using our mouse models. GFP cells in P60 *Ascl1*$^{GFP/+}$ brains showed intense signals in a discrete line along white tracts throughout the corpus callosum (Fig. 5.9A), while weaker signals from scattered cells were detected in gray matter such as the thalamus (Fig. 5.9H). In the corpus callosum, the GFP cells mainly formed clusters in a narrow line that co-label with Ascl1, validating the use of GFP in the knockin to characterize these cells (Fig. 5.9B). These GFP cells co-labeled with BrdU incorporation and Sox2 identifying them as proliferating neural progenitor cells (Fig. 5.9C,D). Restriction

of the GFP cells in a specific region within the corpus callosum suggests it identifies an adult oligodendrogenic region that has not been previously characterized. Furthermore, the GFP cells are partially overlapping with the OPC markers PDGFRα, Olig2 or Sox10, but not with the mature oligodendrocyte marker APC (Adenomatous polyposis coli), indicating Ascl1 is present in progenitors before the known early oligodendrocyte markers are detected (Fig. 5.9E-G, data not shown). In contrast, in gray matter regions such as the thalamus the Ascl1-GFP cells do express the OPC markers PDGFRα, Olig2, and Sox10, but not the mature oligodendrocyte marker APC (Fig. 5.9I-L). This contrast suggests Ascl1-GFP cells are marking OPCs at different developmental stages in the adult brain depending on the environmental context.

To follow the Ascl1-lineage cells over time in the OPCs, I analyzed YFP cells outside the neurogenic zones in the *Ascl1-CreER^{TM};R26R-stop-YFP* animals induced with tamoxifen at P60. Five days after tamoxifen administration, YFP cells were detected in the white matter including corpus callosum, fimbria and internal capsule, and in gray matter regions including the hippocampal formation and thalamus (Fig. 5.10A-C, and data not shown). Comparison between GFP and YFP cells in the corpus callosum of *Ascl1$^{GFP/+}$* and *Ascl1-CreER^{TM};R26R-stop-YFP* mice, respectively, gives an estimated recombination efficiency for Cre of 13%. Regardless of their location in the brain, almost all YFP cells co-expressed PDGFRα, Olig2, and Sox10 (96%) indicating they are in the oligodendrocyte lineage (Fig. 5.10D-F, shown here in thalamus but also seen in corpus callosum). Thus, the subpopulations of GFP cells identified in the corpus callosum of *Ascl1$^{GFP/+}$* that were negative for Olig2 and Sox10, began expressing these oligodendrocyte lineage markers within 5 days of Ascl1 expression. Notably, Ascl1 is transient in this lineage since at 5 days after tamoxifen treatment, there was little overlap in YFP and Ascl1 (Fig. 5.10A, 1% n=91 cells counted). Many YFP cells were observed as doublets, suggesting they had recently divided (Fig.

5.10C). The Ascl1-lineage marked cells had not progressed to a mature oligodendrocyte since only a small fraction of YFP cells (4%) co-labeled with APC (Fig. 5.10G). Together these results illustrate that Ascl1 is transiently expressed in early OPCs that are actively differentiating.

To determine whether the Ascl1-lineage cells progress to mature oligodendrocyte, I examined the YFP cells 30 days after tamoxifen administration. Comparison of the YFP cells in the corpus callosum and thalamus revealed that the OPCs in each region mature at different rates. In the corpus callosum, 58% of the Ascl1-lineage cells matured into differentiated oligodendrocytes and co-label with Sox10 and APC (Fig. 5.10H-J,N), whereas in the thalamus only 20% of the cells co-labeled with APC (Fig. 5.10K-M,N). Previous studies have suggested that OPCs in the adult CNS are arrested at a precursor stage as assessed by retroviral infection or BrdU birthdating (Gensert and Goldman, 1997; Dawson et al., 2003). This does not appear to be true for the Ascl1 expressing OPCs in the white matter tract since a majority of the OPCs matured into oligodendrocytes. Even in thalamus the OPCs are maturing but not as efficiently as in the corpus callosum. *Ascl1*$^{CreERT2/+}$ also labels adult OPCs in the corpus callosum or the fimbria, confirming the results with the BAC transgenic. In contrast, *Ascl1*$^{CreERT2/+}$ did not label OPCs in the gray matter (data not shown). It is possible that the levels of Cre expression in the gray matter OPCs is too low, and thus, these cells are missed by *Ascl1*$^{CreERT2/+}$. Recombination efficiency by the BAC Tg *Ascl1-CreER*^{TM} is generally higher so the gray matter OPCs are detected (See also Chapter three).

Previous studies in the developing spinal cord and neonatal brain placed Ascl1 in neuronal and oligodendrocytic lineages but not astrocytic lineages (Parras et al., 2004; Battiste et al., 2007). Consistent with these embryonic studies, the adult Ascl1-lineage cells do not contribute to the astrocyte lineage since overlap with YFP and the astrocyte marker GFAP or glutamine synthetase was

not detected (Fig. 5.11). Thus, even in the adult neural progenitor cells, Ascl1-lineage is restricted to progenitors to neurons and oligodendrocytes but not astrocytes.

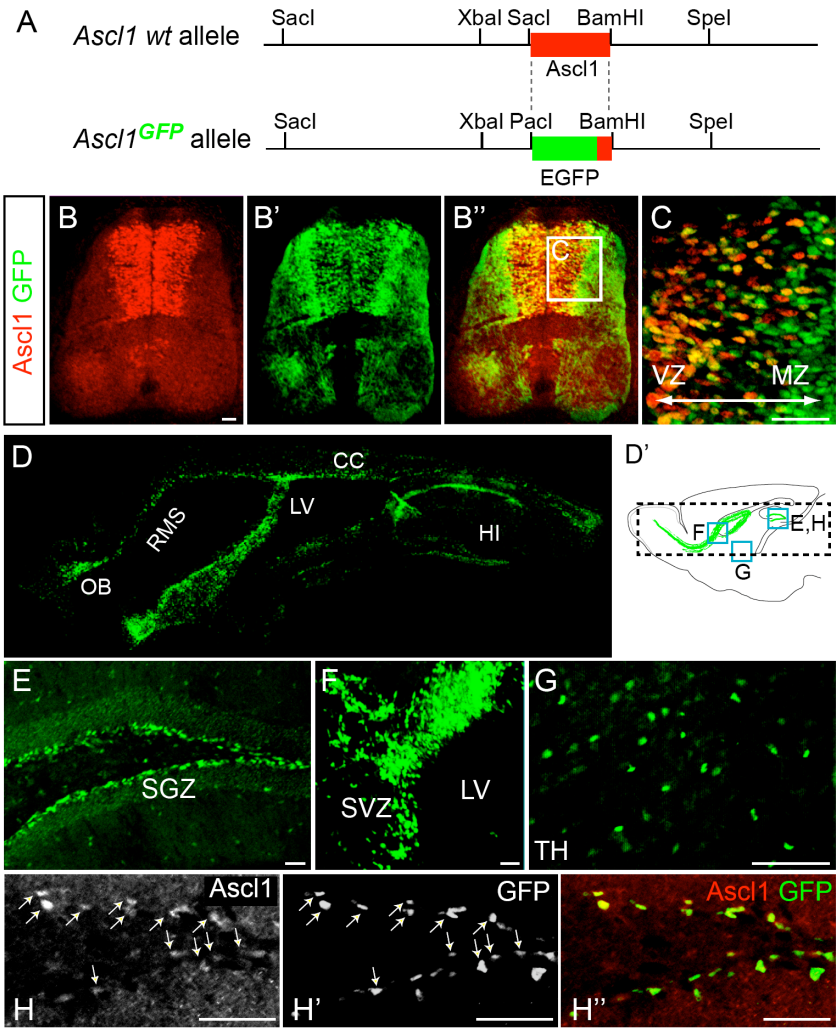

Figure 5.1. Ascl1-GFP expression in the adult brain.
(A) Ascl1-GFP knockin mouse has replaced one allele of Ascl1 coding sequence with that of a nuclear-localized GFP reporter (Leung et al., 2007). (B-H'') are images from immunofluorescence of *Ascl1*$^{GFP/+}$ mice (Ascl1, red; GFP, green). (B-B'') transverse section of a mouse E11.5 neural tube. (C) Higher magnification of (B'') illustrating GFP persists longer than Ascl1. Ascl1 is restricted to the ventricular zone (VZ) while GFP is also present in the mantle zone (MZ). (D) Sagittal view of subcortical regions in mouse forebrain at P60 showing Ascl1-GFP expressing cells are enriched in adult neurogenic regions. (D') Diagram of adult mouse brain indicating where images in (D-H) originated. Ascl1-GFP expression in (E) dentate gyrus, (F) subventricular zone (SVZ) around the lateral ventricle (LV), and (G) thalamus (TH). (H-H'') GFP and Ascl1 are co-expressed

in SGZ of adult hippocampus (arrows). Abbr: CC, corpus callosum; HI, hippocampus; LV, lateral ventricle; MZ, mantle zone; OB, olfactory bulb; RMS, rostral migratory stream; SGZ, subgranular zone; SVZ, subventricular zone; TH, Thalamus; VZ, ventricular zone. Scale bars: 40 μm.

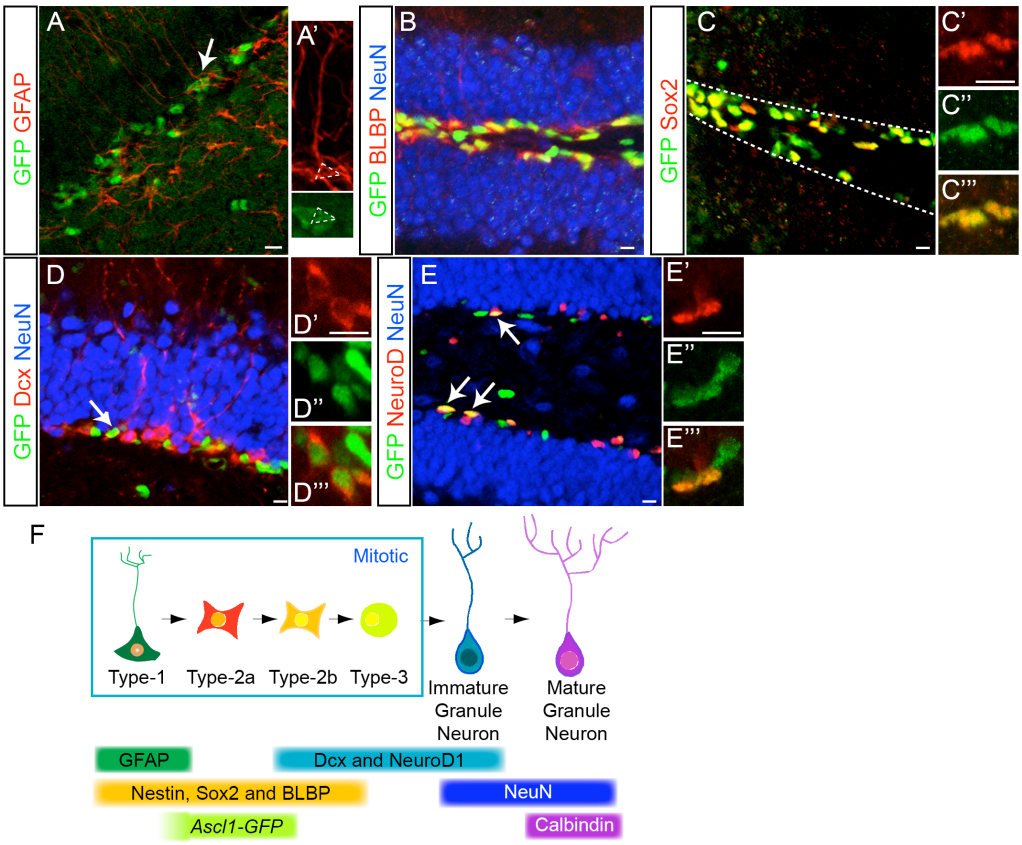

Figure 5.2. Ascl1-GFP cells identified as type-2 cells in the adult hippocampal neurogenic lineage.
The dentate gyrus from P60 *Ascl1*$^{GFP/+}$ mice was examined by immunofluorescence for GFP (A-E, green), GFAP (A, red), BLBP (B, red), Sox2 (C, red), Dcx (D, red), NeuroD (E, red) and NeuN (B,D,E, blue), stage specific markers for cells undergoing neurogenesis in the adult hippocampus (see diagram, G). Higher magnification showing overlap of GFP with Sox2 (C-C''), Dcx (D-D'') and NeuroD (E-E'') are shown. (F) Characterization of the Ascl1-GFP cells places the majority at the Type-2a cell stage according to a current hippocampal neurogenesis model (Steiner et al., 2006). Dashed line indicates SGZ of the DG. Scale bars: 10 µm.

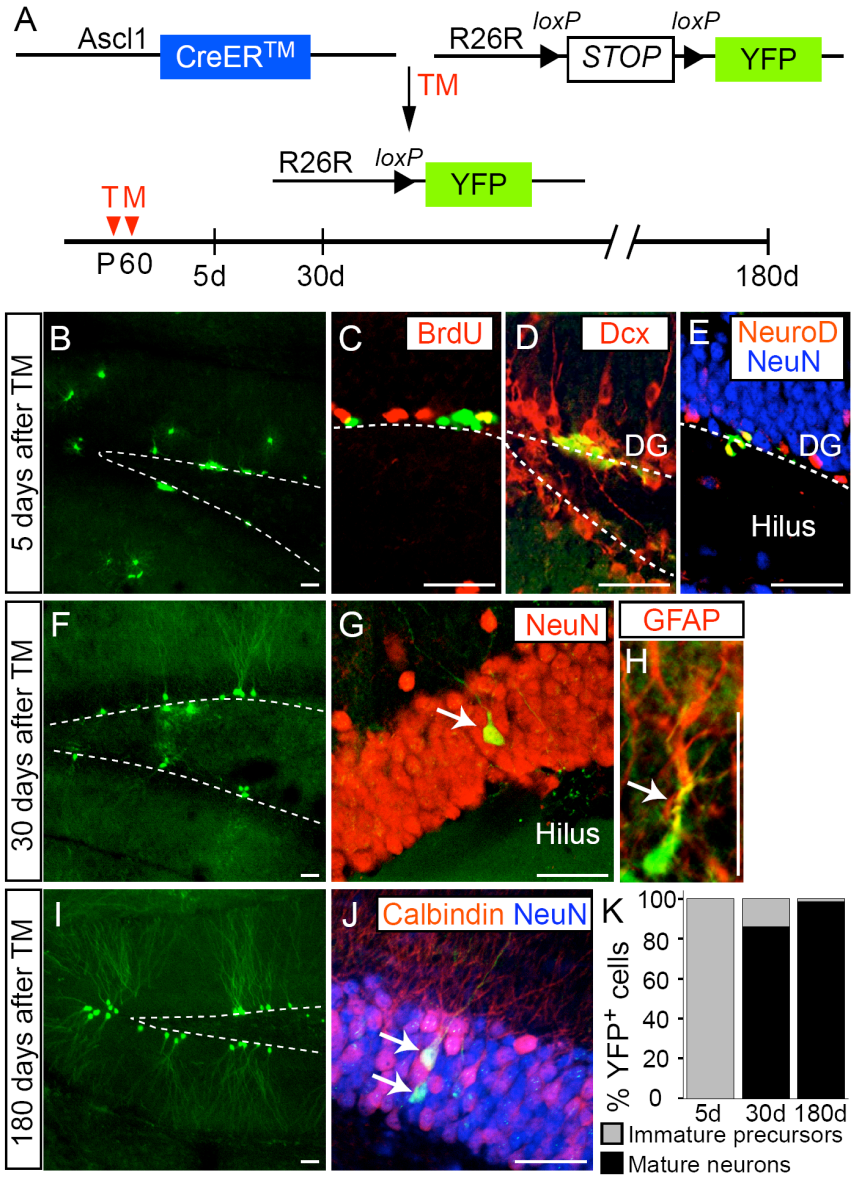

Figure 5.3. Ascl1-lineage cells become mature granule cells in the adult hippocampus.

(A) Diagram illustrating the strategy for in vivo fate mapping of the Ascl1-expressing cells in adult brain. P60 mice carrying alleles *Ascl1-CreER*^{TM} (a tamoxifen inducible Cre recombinase expressed from the Ascl1 locus in a BAC transgene) and *R26R-stop-YFP*

were treated with tamoxifen (TM) and brains were harvested 5, 30, or 180 days later. (B-E) 5 days after tamoxifen treatment YFP (green) is detected in the SGZ of the dentate gyrus (DG) (B). Only few YFP cells incorporate BrdU after a 2 hour BrdU pulse (C). In contrast, most YFP cells co-label with Dcx (D) and NeuroD (E), both Type-2b/Type-3 markers. (F-H) 30 days after tamoxifen treatment YFP cells are found in the granule cell layer in the DG, have morphology of mature granule neurons, and co-label with the neuronal marker NeuN (G, arrow). A few YFP cells elicit stem cell like morphology and co-label with GFAP (H, arrow). (I-J) 180 days after tamoxifen treatment YFP cells are still present in the granule cell layer, have extensive dendritic arborization, and co-label with mature granule cell markers NeuN (blue) and Calbindin (red) (J, arrows). (K) Quantification of the percentage of YFP cells identified as immature precursors or mature neurons at the three different timepoints after TM. Dashed line indicates the SGZ of the DG. Abbr: DG, dentate gyrus; TM, tamoxifen. Scale bars: 40 μm.

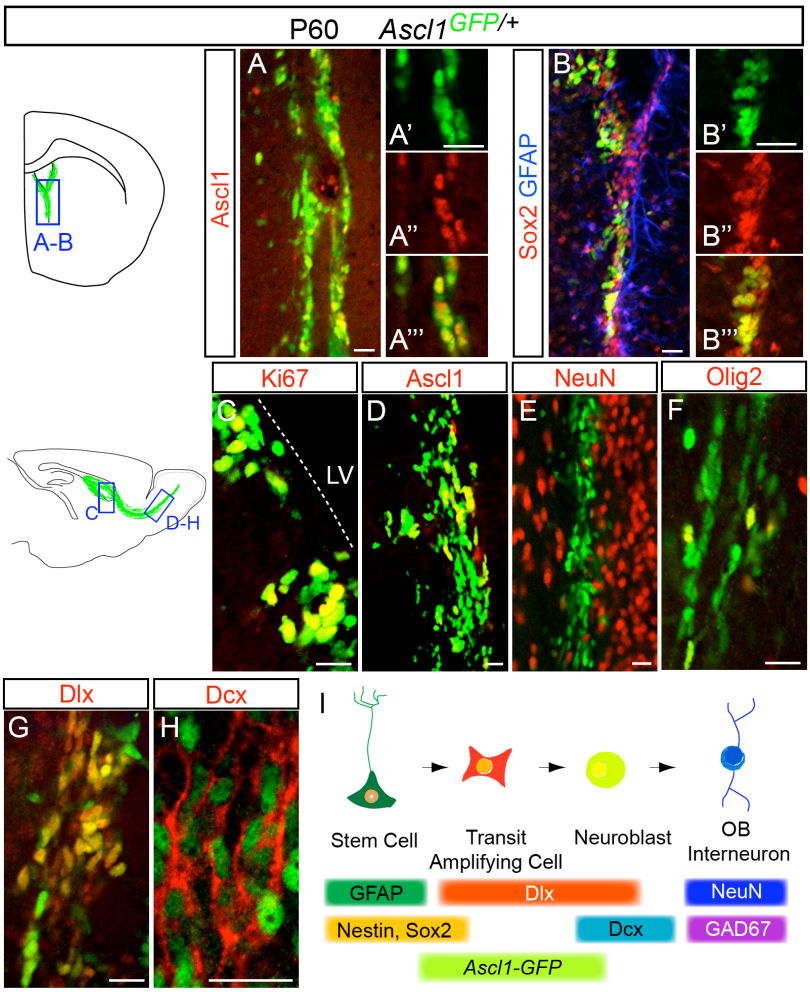

Figure 5.4. Ascl1-GFP cells are transit amplifying cells or neuroblasts in the subventricular zone (SVZ) and rostral migratory stream.
(A-H) Immunofluorescence in P60 *Ascl1*$^{GFP/+}$ brains showing GFP (green) co-labeled with Ascl1 (A,D), Sox2 (B), GFAP (B), Ki67 (C), NeuN (E), Olig2 (F), Dlx (G), and Dcx (H). GFP and Ascl1 overlap in the SVZ and RMS (A-A''', D, yellow cells). GFP also overlaps with Ki67, Sox2, Olig2, Dlx, and Dcx but not GFAP or NeuN identifying Ascl1-GFP cells as transit amplifying/neuroblast cells in this neurogenic region. (I) Ascl1-GFP is placed in transit amplifying cell or neuroblasts using a current model for gene expression staging of olfactory bulb neurogenesis (Doetsch, 2003). Diagrams depict the SVZ in a coronal view, or the SVZ and RMS in a sagittal view of an adult brain showing the location of the GFP cells and regions imaged in (A-H). Abbr: LV, lateral ventricle. Scale bar: 20 μm.

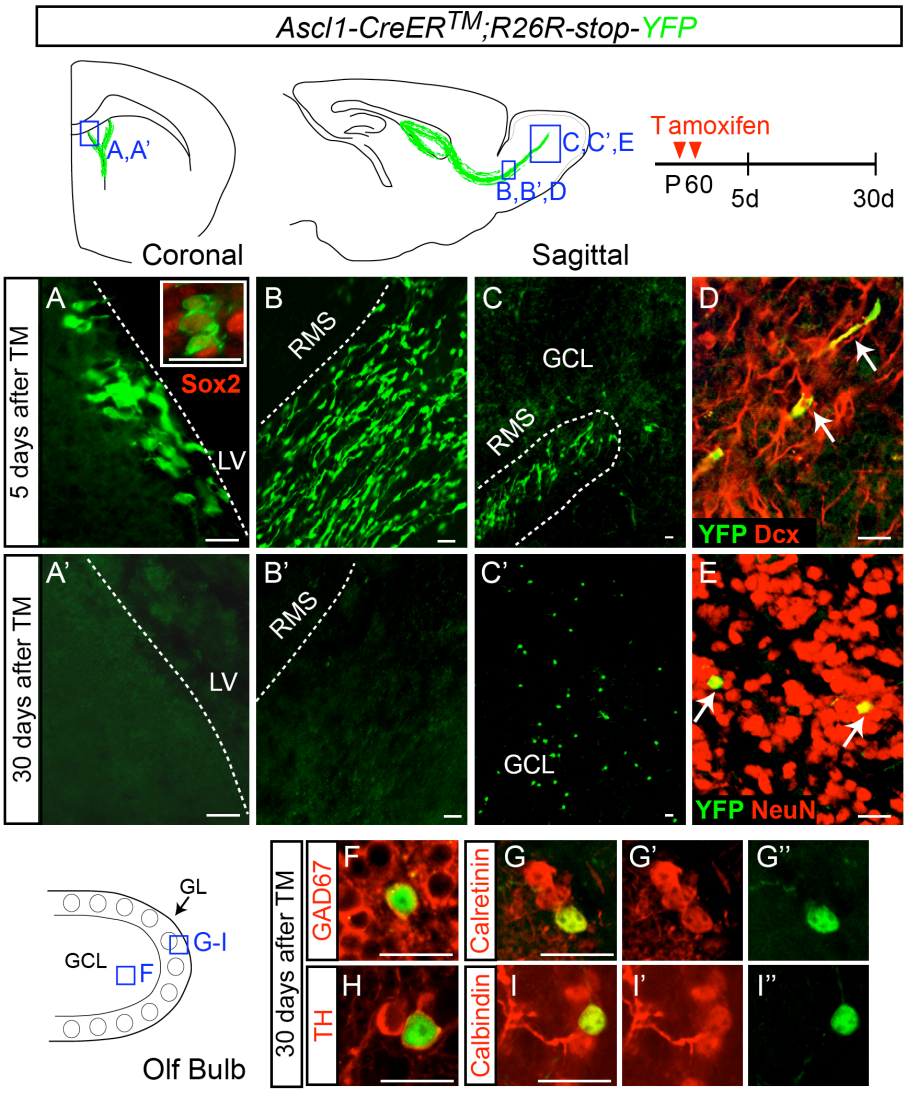

Figure 5.5. Ascl1-lineage cells become interneurons in granule and periglomerular layers of the olfactory bulb.
Immunofluorescence of *Ascl1-CreER^{TM};R26R-stop-YFP* mouse brain sections harvested 5 or 30 days after tamoxifen administration at P60. (A-D) 5 days after tamoxifen treatment some YFP cells (green) are located near the SVZ (A) and co-label with Sox2 (A, red, inset), but most YFP cells were found within the RMS (B,C) and co-label with

the neuroblast marker Dcx (D, arrows). In contrast, 30 days after tamoxifen treatment no YFP cells were detected around the SVZ or RMS (A', B'). At this time the YFP cells were found in the olfactory bulb (C') and express the mature neuronal marker NeuN (E, arrows). (F-I) Most YFP cells in the olfactory bulb 30 days after tamoxifen treatment localized to the GCL where newly generated neurons are homogenously GABAergic shown here as overlap with GAD67 (F). A much smaller proportion of YFP cells were located near the GL in diverse interneuron types overlapping with Calretinin (G), TH (H) or Calbindin (I). Diagrams depict SVZ in a coronal section, or SVZ and RMS, or olfactory bulb in a sagittal section of an adult mouse brain. Boxes indicate the location where images were taken. Abbr: GCL, granular cell layer; GL, glomerular layer; LV, lateral ventricle; RMS, rostral migratory stream; TH, tyrosine hydroxylase; TM, tamoxifen. Scale bars: 20 μm.

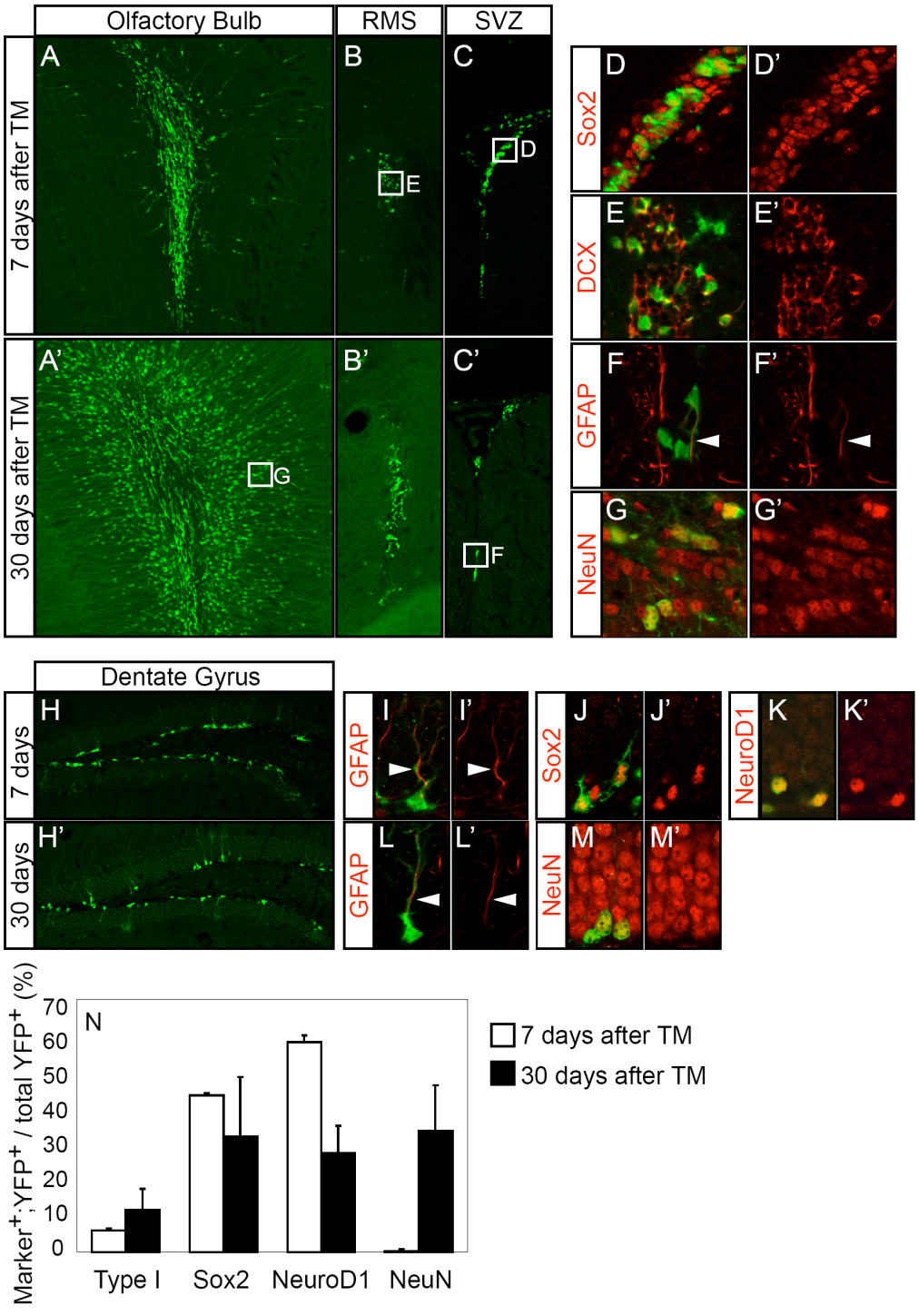
Olfactory Bulb
RMS
SVZ
7 days after TM
30 days after TM
A
B
C
D
E
A'
B'
C'
G
F
Sox2
DCX
GFAP
NeuN
D'
E'
F'
G'
Dentate Gyrus
7 days
30 days
H
H'
I
I'
J
J'
K
K'
L
L'
M
M'
NeuroD1
N
Marker+;YFP+ / total YFP+ (%)
70
60
50
40
30
20
10
0
Type I
Sox2
NeuroD1
NeuN
7 days after TM
30 days after TM

Figure 5.6. *Ascl1*^{CreERT2} reveals the dynamics of Ascl1^{+} neural stem cell lineage development in the SVZ and the SGZ of adult hippocampus.
Immunofluorescence in *Ascl1*$^{CreERT2/+}$*;R26R-stop-YFP* mouse brain sections harvested 7 or 30 days after tamoxifen administration. (A-G') lineage tracing of Ascl1 derived cells along SVZ-RMS-OB pathway. 7 days after tamoxifen administration, most YFP cells were located in the SVZ (C) or along the RMS (A-B) and express progenitor marker Sox2 (D-D') or neuroblast marker Dcx (E-E'), respectively. 30 days after tamoxifen administration, YFP cells mature into neurons in the granule cell layer of the dentate gyrus or the periglomerular layer of the olfactory bulb (A', G-G', and data not shown). In addition, many YFP^{+} cells remain as progenitors in the RMS or SVZ (B'-C'). In the SVZ, a few YFP cells express the neural stem cell marker GFAP (F). (H-N) Lineage tracing of Ascl1 derived cells in the SGZ of hippocampus. 7 days after tamoxifen administration, 6% of YFP cells express GFAP or have a Type-1 stem cell-like morphology. 44% of YFP cells express Sox2 and 58% of YFP cells express NeuroD1, whereas no NeuN^{+} YFP mature neurons are detected. 30 days after tamoxifen administration, 34% of YFP cells express NeuN, a mature neuronal marker. 32% or 27% of YFP cells express Sox2 and NeuroD1, respectively. (N) Quantification of the percentage of YFP cells co-labeled with Sox2, NeuroD1, or NeuN 7 or 30 days after tamoxifen administration.

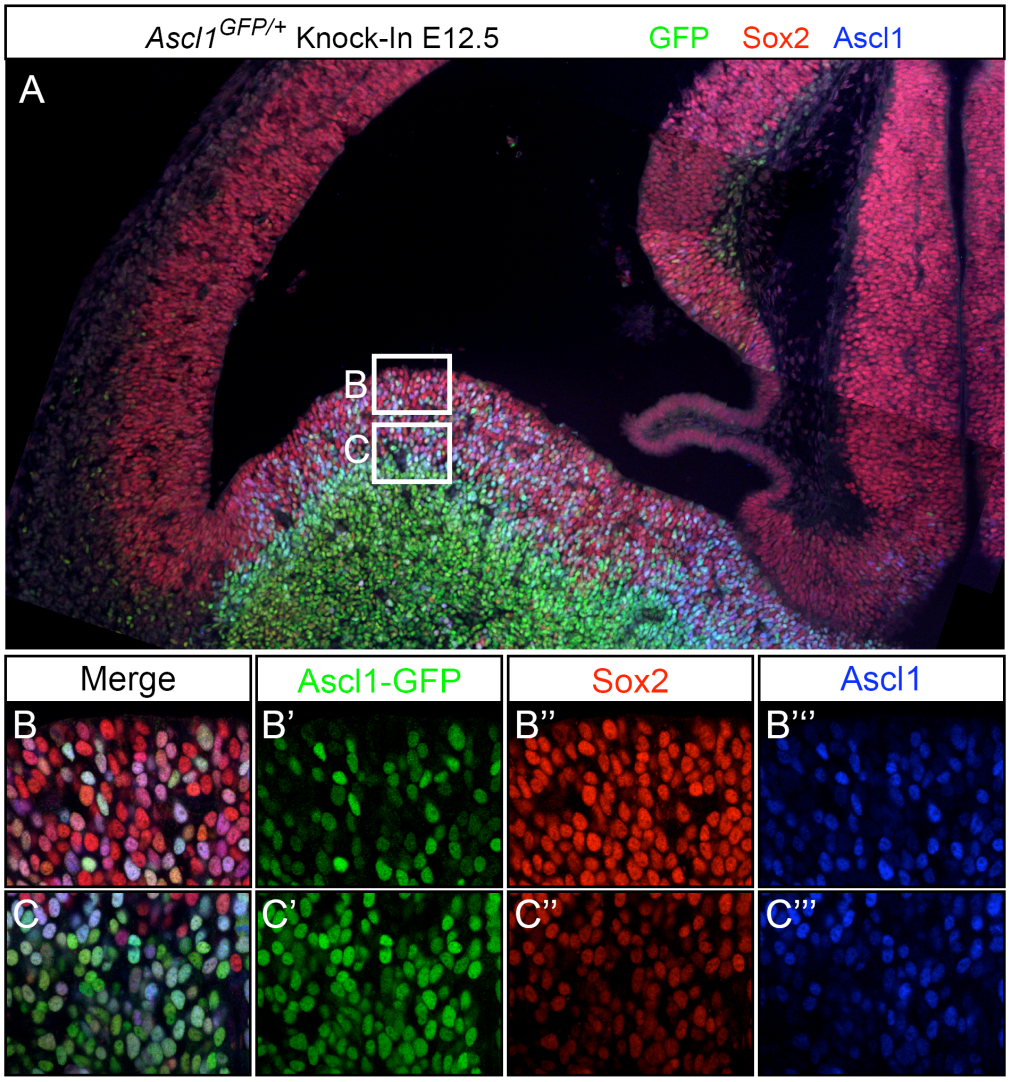

Figure 5.7. Ascl1 expressing cells in the embryonic germinal zone of ventral telencephalon are stem like/progenitor cells.
Immunofluorescence in the ganglion eminence of *Ascl1*$^{GFP/+}$ embryos at E12.5. In both apical and basal regions of the ventricular zone (B-C'''), a subset of Sox2^{+} stem cell/early progenitors express Ascl1-GFP or Ascl1.

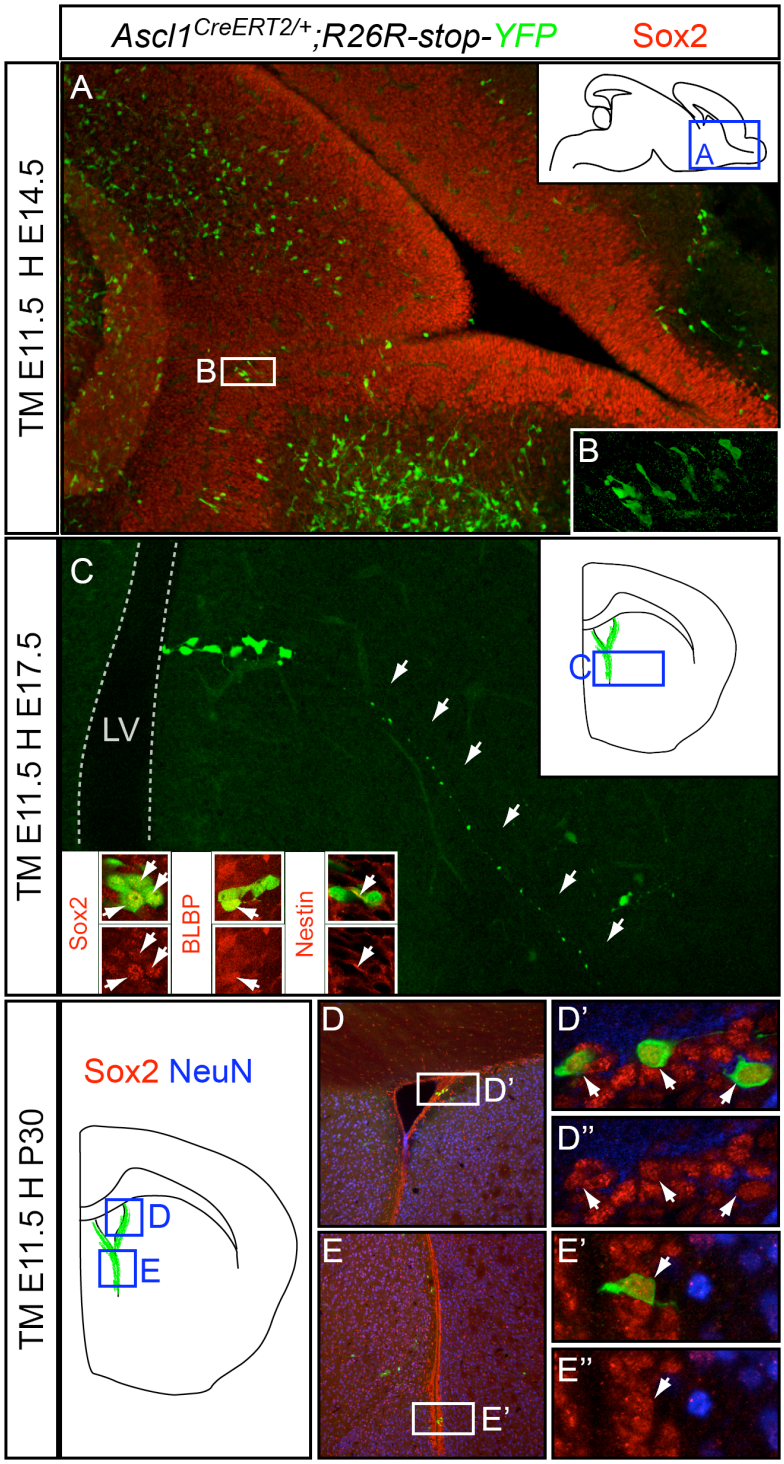
Ascl1CreERT2/+;R26R-stop-YFP
Sox2
TM E11.5 H E14.5
A
B
TM E11.5 H E17.5
C
LV
Sox2
BLBP
Nestin
TM E11.5 H P30
Sox2 NeuN
D
E
D'
D''
E'
E''

Figure 5.8. Some Ascl1 expressing embryonic progenitors develop into adult neural progenitors in the SVZ.
Immunofluorescence of *Ascl1*$^{CreERT2/+}$*;R26R-stop-YFP* brain sections after tamoxifen administration at E11.5. (A-B) 3 days after tamoxifen administration, some YFP cells remain in the ventricular zone (A). They show radial glia like morphology such as end feet structures (B). (C) 6 days after tamoxifen administration, clones of YFP^{+} cells are detected around the subventricular zone. Some YFP cells have radial glia like process reaching to the pia mater (arrows). (Inset) YFP cells express stem cell/progenitor markers such as Sox2, BLBP, or Nestin (arrows). (D-E'') In a P30 brain after tamoxifen administration at E11.5, Sox2^{+} (red) and NeuN^{-} (blue) YFP^{+} cells are present in the SVZ. Abbr: LV, lateral ventricle.

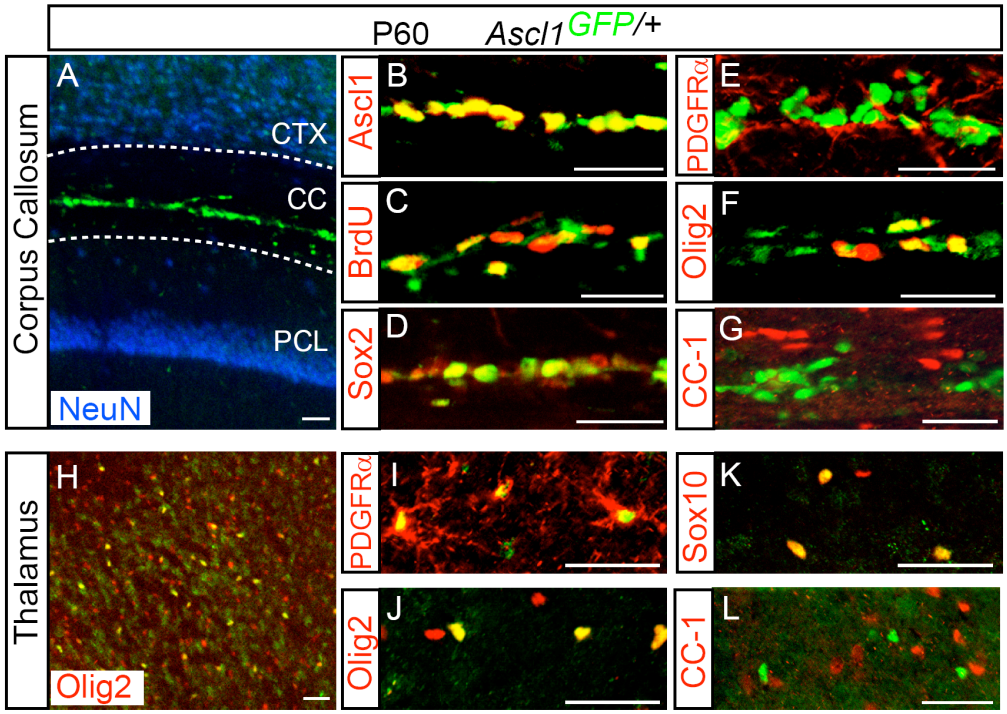

Figure 5.9. Oligodendrocyte restricted progenitors (OPCs) identified by Ascl1-GFP outside neurogenic niches in adult brain.
Immunofluorescence in P60 *Ascl1*$^{GFP/+}$ brains showing GFP (green) in corpus callosum with NeuN (A), Ascl1 (B), BrdU incorporation (C), Sox2 (D), PDGFRα (E), Olig2 (F), and APC (G), or in thalamus with Olig2 (H,J), PDGFRα (I), Sox10 (K), and APC (L). In the corpus callosum, Ascl1-GFP cells are largely restricted to a narrow line on the ventral side that reliably co-express Ascl1, mostly co-label with neural progenitor markers such as Sox2, have minor overlap with OPC markers PDGFRα, Olig2, and Sox10, and rarely overlap with the mature oligodendrocyte marker APC. Ascl1-GFP cells in the thalamus co-label with the OPC markers PDGFRα, Olig2, and Sox10 but not APC. Abbr: CC, corpus callosum; CTX, neocortex; PCL, pyramidal cell layer of hippocampus. Scale bars: 40 μm.

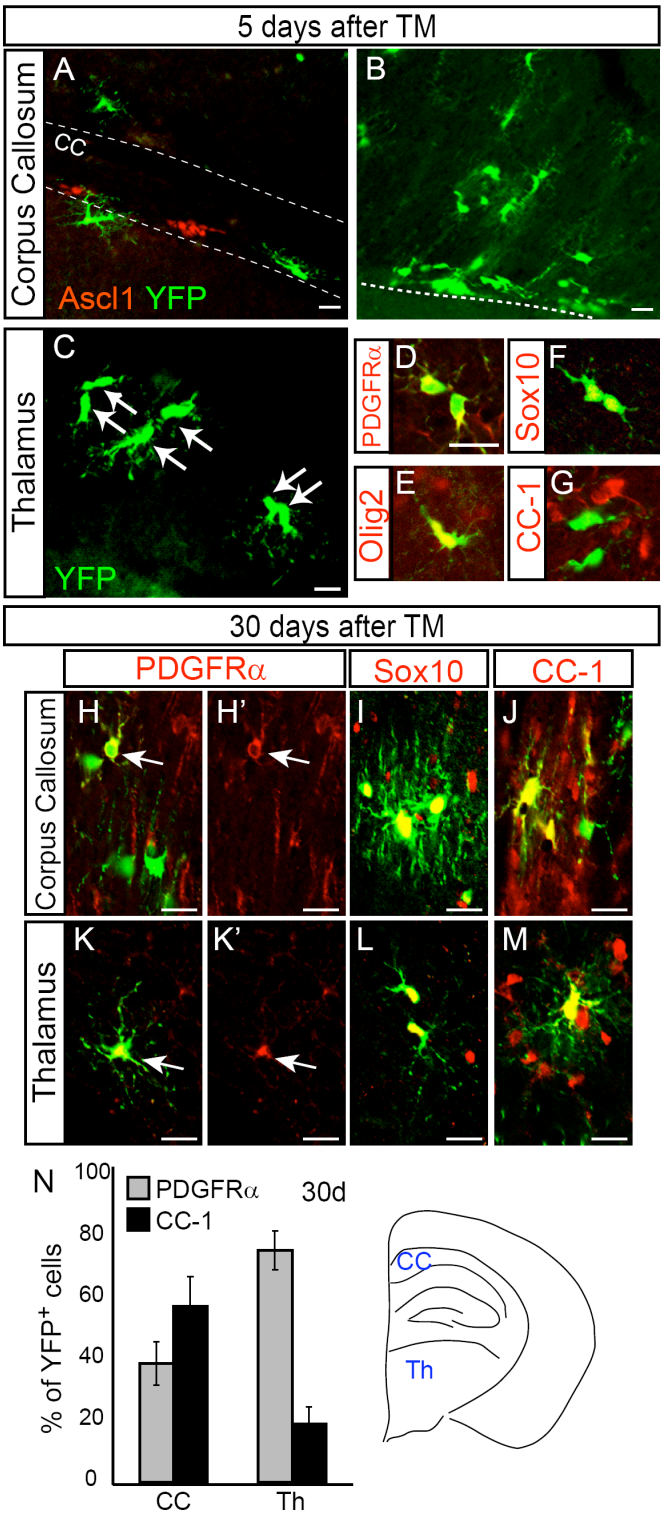
5 days after TM
Corpus Callosum
A
cc
Ascl1 YFP
B
Thalamus
C
YFP
PDGFRα
D
Sox10
F
Olig2
E
CC-1
G
30 days after TM
PDGFRα
Sox10
CC-1
Corpus Callosum
H
H'
I
J
Thalamus
K
K'
L
M
N
100
80
60
40
20
0
% of YFP+ cells
PDGFRα
CC-1
30d
CC
Th
CC
Th

Figure 5.10. Ascl1-lineage OPCs become mature oligodendrocytes in the adult brain.
Immunofluorescence of *Ascl1-CreER^{TM};R26R-stop-YFP* mouse brain sections harvested 5 or 30 days after tamoxifen administration at P60. (A-G) 5 days after tamoxifen administration YFP cells (green) in corpus callosum no longer co-label with Ascl1 and they appear to spread from the ventral surface (A,B).YFP cells are often found as doublets, indicating they were recently dividing, and illustrated here in thalamus (C, arrows). In both the corpus callosum and thalamus, YFP cells co-label with the OPC markers Olig2 (D), PDGFRα (E) and Sox10 (F), but not the mature oligodendrocyte marker APC (G). (H-N) 30 days after tamoxifen administration, in both corpus callosum (H-J) and thalamus (K-M), YFP cells co-label with OPC markers PDGFRα (H,H',K,K') and Sox10 (I,L), but also with the mature oligodendrocyte marker APC (J,M). (N) Quantification of the percentage of YFP cells co-labeled with the OPC marker PDGFRα or the mature oligodendrocyte marker APC in both corpus callosum (CC) and thalamus (Th) 30 days after tamoxifen. Diagram depicts the corpus callosum (CC) and thalamus (Th) in adult coronal brain where images were taken. Scale bars: 20 μm.

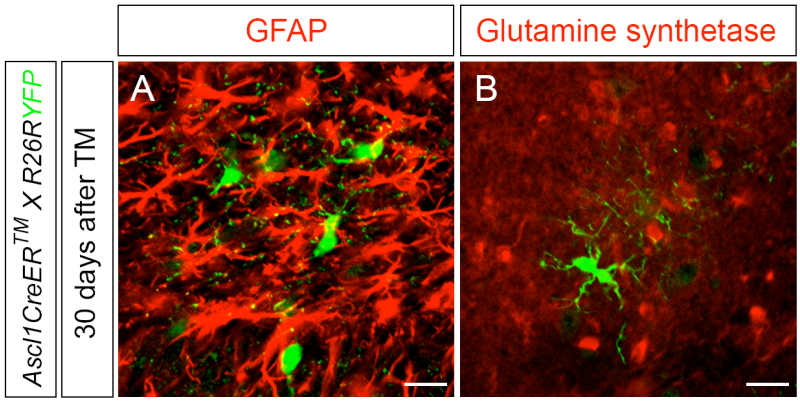

Figure 5.11. Ascl1 lineage cells do not give rise to astrocytes.
Ascl1 lineage cells do not express astrocyte marker GFAP (A) in hippocampal formation or glutamine synthetase (B) in substantia nigra after 30 days of Ascl1 expression. Scale bar: 20 μm.

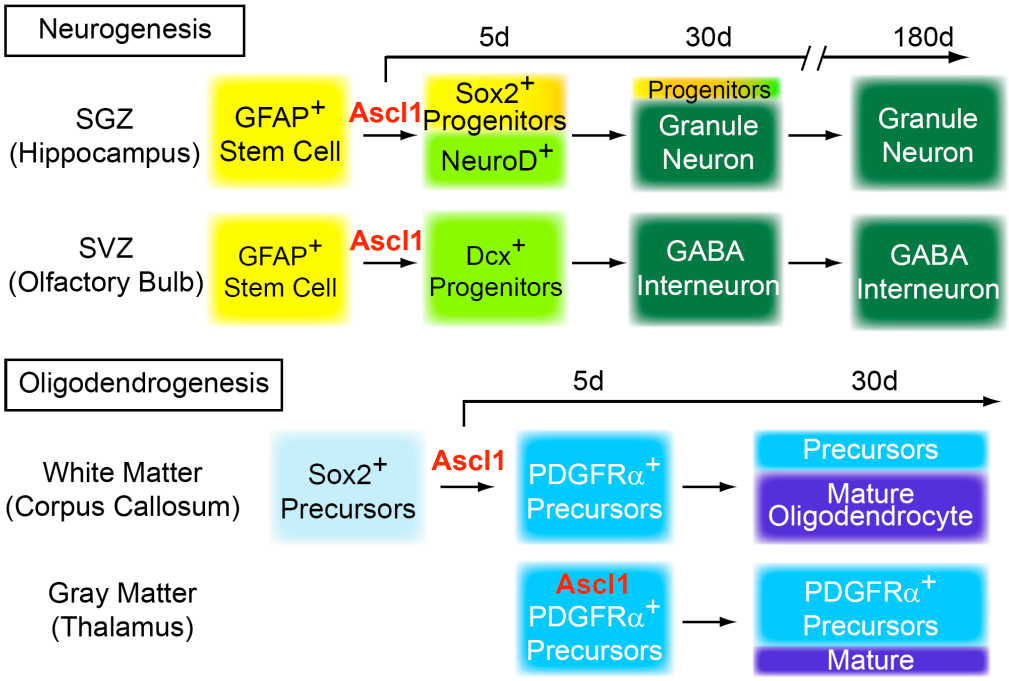

Figure 5.12. Model of the dynamics of maturation of Ascl1 lineage cells during adult neurogenesis and oligodendrogenesis.
In both adult neurogenesis and oligodendrogenesis, Ascl1 expression is transient. Although developmental stages of cells expressing *Ascl1* are slightly different, the onset of *Ascl1* expression parallels the transition from quiescent stem cells or precursors to immature progenitors. In addition, the rates at which each of these lineages mature are varied. For adult neurogenesis, the terminal differentiation of all Ascl1 lineage cells from the SVZ is completed within 30 days, whereas the maturation of all Ascl1 lineage cells from the SGZ in the hippocampus takes longer. In oligodendrogenic lineages, Ascl1 expressing precursors in white matter mature more rapidly than Ascl1 expressing precursors in gray matter.

DISCUSSION

Ascl1 is unique in that it is a transcription factor with essential regulatory functions in directing both neurogenesis and oligodendrogenesis. In both lineages, Ascl1 is transient and identifies early neural precursor cells. These characteristics of expression are seen in the adult brain as well as in the developing neural tube, highlighting molecular similarities between these temporally distinct processes. In the adult brain I have defined the profile of Ascl1 expressing neural precursor cells and subsequently followed the lineage of these cells in four distinct brain regions (Fig. 5.12). These regions will be discussed in turn and include the two regions giving rise to new neurons, the SGZ of the dentate gryrus and the SVZ/RMS supplying neurons to the olfactory bulb, and two regions with distinct OPCs, the corpus callosum as a white matter tract and the thalamus representing a subcortical gray matter region.

Ascl1 lineage in adult neurogenesis in the dentate gyrus of the hippocampus

Using the *Ascl1*$^{GFP/+}$ mouse, our study identifies the majority of Ascl1 cells as Type-2a cells with a minority being Type-1 cells in the adult dentate gyrus. According to a current model of adult hippocampus neurogenesis, GFAP, Sox2, and Nestin expressing neural stem cells (Type-1 cells) differentiate into neural progenitor cells expressing NeuroD and Dcx (Type-2b or 3 cells). Recent reports place transcription factors, Tbr2 and Neurog2, in the Type-2b or 3 cells as well, a stage after Ascl1 expression (Hevner et al., 2006; Ozen et al., 2007). Ascl1 is a unique marker for Type-2a cells, and in particular it marks cells that are actively transitioning from progenitor to differentiated neuron. Long-term fate mapping of Ascl1 progenitors in the *Ascl1-CreER*^{TM} transgenic mice also supports this idea, showing that by six months almost all of the progeny in the hippocampus have matured into granule neurons. In contrast, using the *Ascl1*$^{CreERT2/+}$ knockin line, or

similar paradigms with inducible Cre under the control of Gli1 (Ahn and Joyner, 2005) or Nestin (Lagace et al., 2007), stem cell populations are not depleted and generate neurons continuously. Although Ascl1 appears to largely mark Type-2a cells, there is some overlap detected in $GFAP^+$ cells. In addition, it has been suggested that $GFAP^+$ SGZ cells can be divided into cells having long-term self-renewal ($GFAP^+/Ascl1^-$) and cells with more limited dividing potential ($GFAP^+/Ascl1^+$) (Seki et al., 2007). It still remains a question as to whether $GFAP^+/Ascl1^+$ stem cells show comparable dynamics of lineage development to that of Gli1 or Nestin-$CreER^{T2}$ models, particularly in the long term self renewal property.

Ascl1 lineage in adult neurogenesis in the SVZ/RMS and the generation of olfactory bulb interneurons

Ascl1 transiently marks transit amplifying cells and neuroblasts in adult olfactory bulb neurogenesis (Kohwi et al., 2005). In this lineage, neural stem cells residing around the lateral ventricle wall migrate along the rostral migratory stream (RMS) to the olfactory bulb as they differentiate. GFP cells in the *Ascl1*$^{GFP/+}$ mouse show broad but discrete expression of GFP along this pathway. These cells are $Sox2^+$, $Ki67^+$ or Dcx^+, indicating their identity as amplifying neural progenitor cells, consistent with previous reports using a transgenic mouse with Ascl1 regulatory sequences driving LacZ (Parras et al., 2004). The *Ascl1-CreER*^{TM} mouse allowed us to determine the timing of differentiation and to examine if Ascl1 cells continue to self-renew. Strikingly, 30 days after Ascl1 expression, the Ascl1 lineage cells were located in the olfactory bulb, with none found in the SVZ or RMS demonstrating all Ascl1 lineage cells detected in this paradigm differentiate and none stay as stem/progenitor cells. In contrast, in similar paradigms but using *Ascl1*^{CreERT2} or *Nestin* (Kuo et al., 2006) or *Gli1* (Ahn and Joyner, 2005) to drive the inducible Cre, the stem cell and progenitor populations maintain the lineage

marker over time in SVZ and RMS. Clear differences between *Ascl1-CreER*^{TM,}, *Ascl1*^{CreERT2} and *Nestin*- or *Gli1CreER*^{T2} suggest that comparisons between these model systems will elucidate novel information (ie via gene expression profiling) regarding stem and transit amplifying cells at distinct stages during adult neurogenesis.

Ascl1 lineage neurons in adult hippocampus and olfactory bulb have diverse neuronal sub-types

During embryogenesis, Ascl1 functions in neuronal differentiation and sub-type specification for diverse cell types throughout the nervous system. In the dorsal spinal cord, Ascl1 functions in specifying glutamatergic interneuron populations dI3 and dI5 (Helms et al., 2005). In telencephalon development, Ascl1 functions with Dlx2 to generate the GABAergic neurons that migrate to the cortex (Fode et al., 2000; Parras et al., 2002; Long et al., 2007). And in the peripheral nervous system, Ascl1 biases neural crest cells to the autonomic lineage rather than the sensory (Perez et al., 1999). Thus, although Ascl1 appears to play a role in neuronal sub-type specification, the specific neuronal subtypes formed depend on the cellular environment and developmental history. This concept for Ascl1 function in neuronal specification holds in the adult as well. Ascl1 lineage neurons in the adult hippocampus generate exclusively glutamatergic neurons, with no evidence for Ascl1 progenitors giving rise to GABAergic neurons in this region. In contrast, adult generated Ascl1 lineage cells in the olfactory bulb are all GABAergic neurons with no obvious preference towards individual sub-types distinguished by TH, Calretinin and Calbindin in the glomerular layer.

Ascl1 lineage in adult oligodendrogenesis in the corpus callosum

Analyzing the *Ascl1*$^{GFP/+}$ mouse brain for GFP and endogenous Ascl1, I find many Ascl1 expressing cells in the adult corpus callosum, a wide axon bundle

containing myelinated oligodendrocytes beneath the neocortex. Surprisingly, the Ascl1 population forms a discrete layer of cells along the ventral side of the corpus callosum, a cell pattern that has not been previously characterized. Other oligodendrocyte markers including those considered early markers such as PDGFRα and Olig2 do not show this distinct pattern, but rather are distributed throughout the corpus callosum. This observation suggests that Ascl1 is the earliest OPC marker identified and that the discrete line of Ascl1 cells in the corpus callosum defines an oligodendrogenic niche. Supporting this idea, Ascl1-GFP cells mostly express Sox2, but only partially overlap with early OPC markers such as Olig2 and Sox10, suggesting Ascl1 expression is prior to these markers. Consistent with this, using the inducible Cre system to follow the lineage, the Ascl1 cells have progressed to PDGFRα$^+$ and Sox10$^+$ OPCs within 5 days, with many of these cells appearing as doublets. In addition, in late stage spinal cord embryogenesis, Ascl1 expresses earlier than Sox10 and Olig2 as well (See Chapter three). At E15.5, early precursor cells are lined along ventricular zone and move out as they mature to oligodendrocytes. Within the ventricular zone, there are few Ascl1 cells co-labeled with Olig2/Sox10, whereas in the mantle zone they are more likely to co-express Olig2/Sox10. Therefore, in oligodendrogenic niches, Ascl1 marks OPCs at an earlier stage than Olig2 and Sox10. Furthermore, in the adult, Ascl1 expression is transient and disappears as the other OPC markers remain.

The transient nature of Ascl1 in the OPC lineage makes the *Ascl1-CreER^{TM}* mouse uniquely suited to follow the progression of OPCs as they mature in the adult brain. Oligodendrocyte progenitors marked by *Ascl1-CreER^{TM}* differentiate within 30 days to express APC, a mature oligodendrocyte marker. This indicates Ascl1$^+$ OPCs in the corpus callosum are not quiescent, but actively mature even in an intact non-injured brain. Previous studies using stereotactic injection of retrovirus or BrdU birthdating showed cycling cells in the corpus

callosum only rarely mature into myelinating oligodendrocytes, with the rate of maturation dramatically increased after brain insult (Gensert and Goldman, 1997; Dawson et al., 2003). It is possible that the Ascl1 lineage marked cells in the corpus callosum identify a subset of the cells marked in the previous studies, a subset that is more actively maturing.

Ascl1 lineage in adult oligodendrogenesis in subcortical gray matter: thalamus

Not only white matter but also subcortical gray matter contains widespread Ascl1-GFP cell populations. The Ascl1 marked OPCs in the thalamus already co-express Sox10, Olig2, and PDGFRα, indicating they are committed oligodendrocyte progenitors as previous studies reported (Woodruff et al., 2001). These cells also contrast in their rate of maturation with the Ascl1 OPCs in the corpus callosum in that they mature more slowly and after 30 days the majority remains immature, expressing PDGFRα and not APC. Different antigenic profiles and differentiation rates of Ascl1 lineage OPCs between the white matter tract of the corpus callosum and gray matter of the thalamus suggest Ascl1 is marking different subsets of OPCs. Future studies will be needed to determine whether this also represents a functional distinction.

Concluding Remarks

Here I show the first comprehensive lineage analysis from Ascl1 expressing cells in adult brain. Adult Ascl1 expressing cells share similar molecular characteristics with embryonic counterparts and differentiate into mature neural cells. Along with the previous studies regarding the expression of other transcription factors and signaling molecules such as Notch, Shh and Wnt in adult brain (Stump et al., 2002; Ahn and Joyner, 2005; Hack et al., 2005; Lie et al., 2005; Ozen et al., 2007), this study supports the idea that adult CNS controls neural stem cell

differentiation using similar molecular mechanisms as in the embryonic CNS. Ascl1 is unique as a classII bHLH transcription factor in that it is involved in both adult neurogenesis and oligodendrogenesis, and its expression is restricted within narrow developmental windows in both processes. This characteristic of Ascl1 expression makes the *Ascl1-CreER*^{TM} and *Ascl1*^{CreERT2} mice valuable tools for studying the developmental dynamics of adult born neurons and oligodendrocytes that will contribute insights into underlying causes of brain tumor development and other neurological disorders.

ACKNOWLEDGEMENTS

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CHAPTER SIX

Conclusions and future directions

In this thesis, I delineated neuronal and glia lineages that arise from Ascl1 expressing progenitors using multiple mouse models and comprehensive lineage tracing (Fig. 6.1). Many diverse but discrete cell populations derived from Ascl1 contribute to the adult CNS structure. The lessons that I learned from previous chapters not only contributes to our understanding of the brain and spinal cord formation but also raises many new interesting questions pertaining to Ascl1 biology and vertebrate neural development.

Genetic fate mapping and lineage models

Lineage tracing has been a crucial tool to understand the principle of organogenesis: how a cell or group of cells determine their destines during any developmental process. As a classic example, the complete lineage map of *Caenorhabditis elegans* was achieved by direct observation using electron microscopy (Sulston et al., 1983). To study lineage development in embryos in utero such as mouse or human, methods were classically limited to approaches such as physical mapping with dye injection or direct time lapse observation. With the advent of transgenic mouse technology and site-specific recombinases such as Cre recombinase or Flipase, 'genetic' fate mapping created the opportunity to trace cohorts of cells defined by gene expression to study their behavior and fate throughout development. However, a single clone cannot be followed and analyzed with genetic fate mapping in a non-invasive manner; therefore, lineage models proposed from these strategies await corroboration by other techniques.

In chapter two, I identified two spatially and temporally discrete progenitors expressing Ascl1 that give rise to GABAergic and glutamatergic neurons differentially in the dorsal neural tube. These two different progenitor populations are closely related temporally in the lineage, likely within one or two cell divisions. The lineage dynamics of individual cells at this level of resolution need to be observed in real time using time lapse microscopy. The direct monitoring of clonal cells will provide more insights into understanding gliogenic and adult neurogenic lineages. It still remains to be determined whether oligodendrocytes and astrocytes arise from a common bipotential progenitor or from lineage-restricted progenitors. In a similar manner, it is still not clear whether Ascl1^{+} adult neural progenitors are bipotential for neurons and oligodendrocytes, or if Ascl1 is expressed in lineage restricted progenitors to these diverse cell types.

Undoubtedly the tools generated for the genetic fate mapping studies allow us to address more refined questions about Ascl1 function. First, there are multiple Ascl1 lineages defined in this thesis where the function of Ascl1 is not known including Purkinje cells in the cerebellum, astrocytes, and adult generated neurons and oligodendrocytes. The conditional allele of Ascl1 combined with different Cre driver lines enable one to address Ascl1 function without complications such as earlier developmental defects or lethality. Second, in spinal cord neurogenesis, distinct Ascl1^{+} progenitors for dIL^{A} and dIL^{B} fates implicate their molecular profiles are also different. Thus, although Ascl1 marks both progenitors, its mechanism of action or downstream targets may be different. Interestingly, the expression of Dll1 and Dll3, two known transcriptional targets of Ascl1, show distinct spatial patterns in the neural tube. In contrast to the Ascl1-like expression of Dll1 at E11.5 neural tube, Dll3 expression is more enriched in the lateral edge of the progenitor domain, resembling the pattern seen when dIL^{B} restricted progenitors were labeled. In fact, in the neural tube of transgenic

embryos expressing GFP under the control of the *Dll3* promoter, GFP^+ cells preferentially become dIL^B neurons (data not shown). It would be intriguing to determine if Ascl1 regulates distinct Notch ligand targets in distinct progenitors. Third, a surprising finding from $Ascl1^{CreERT2/+}$ line is that Ascl1 is expressed in stem cells. In both embryonic and adult brain, Ascl1 lineage cells remain in the progenitor domain after their initial Ascl1 expression, and appear to generate clonal progeny. One possibility is that Ascl1 has a function in stem cell maintenance, possibly through balancing the activity of the Notch signaling pathway.

Molecular mechanisms of Ascl1 function

Since its discovery in 1990, Ascl1 has been studied extensively in diverse developmental contexts. Largely based on the phenotypes in *Ascl1* null embryos or overexpression paradigms, Ascl1 is shown to induce neuronal differentiation and play a role in neuronal sub-type specification. In addition, recent studies, including this thesis, reveal that Ascl1 also regulates oligodendrocyte development. However, it still remains to be understood clearly how Ascl1 regulates neurogenesis or gliogenesis at the molecular level. A major hurdle in this area has been the difficulty in identifying co-factors and downstream targets.

Modern techniques have been developed that are allowing researchers to define binding sites for transcription factors throughout the genome. Compiling the transcriptome of $Ascl1^+$ cells can be approached in several distinct but synergistic ways. For example, taking advantage of transgenic lines such as $Ascl1^{GFP/+}$, the expression profiles of Asc1-GFP^+ progenitors can be generated after FACS (fluorescence-activated cell sorting) and the use of Affymetrics microarrays or RNA-Seq deep sequencing. Deep sequencing technologies to identify sequences enriched after chromatin immunoprecipitation with antibodies specific to Ascl1, Chip-Seq, is providing huge advances in the identification of in

vivo transcriptional targets of Ascl1. The molecular differences in Ascl1$^+$ progenitor cells and the genes occupied by Ascl1 can be compared in a variety of ways. The differences in Ascl1$^+$ neuronal progenitors versus Ascl1$^+$ glial progenitors, or Ascl1$^+$ progenitors from the embryo versus the adult brain, may reveal context-specific molecular environments and targets for specific functions.

Ascl1 is one of the most important transcription factors regulating neurodevelopment. This thesis reveals that its contribution to diverse neural cell types and its functions in multiple distinct contexts are far more complex than has been previously appreciated. Future studies based on the experiments and the observations in this thesis would provide fundamental insights into principles of neural cell type diversification at a molecular level.

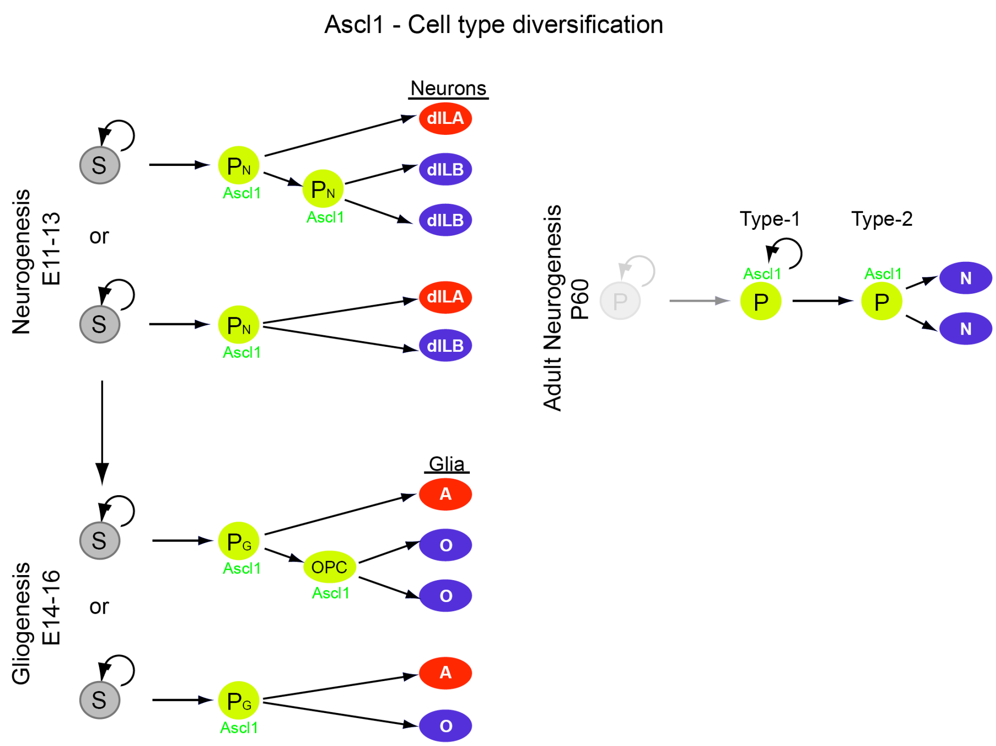
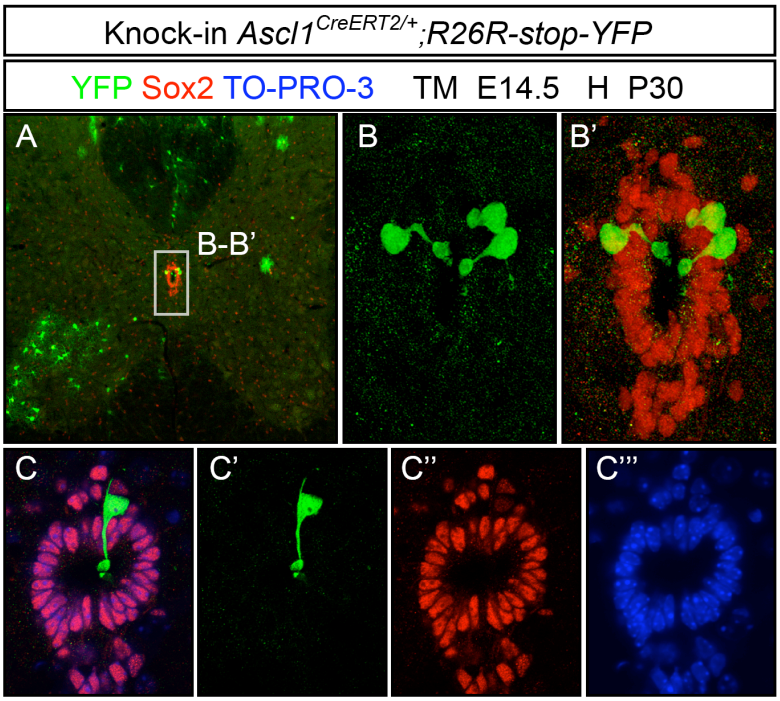


Figure 6.1. Lineage model of neurogenesis and gliogenesis of Ascl1 expressing progenitors in the CNS. A, astrocyte; dILA, dorsal interneuron late A; dILB, dorsal interneuron late B; N, neuron; O, oligodendrocyte; OPC, oligodendrocyte precursor cell; P, progenitor; P_G, glial progenitor; P_N, neuronal progenitor; S, Stem cell.

APPENDIX A

Non-neural lineage of Ascl1 in the spinal cord



In *Ascl1*$^{CreERT2/+}$*;R26R-stop-YFP* animals after E14.5 tamoxifen administration, I observed that Ascl1 lineages also contribute to the ependymal layer surrounding the central canal of the adult spinal cord. Previous studies showed that Nkx6.1 expressing ventral neuroepithelium generate ependymal cells (Richardson et al., 1997; Fu et al., 2003). Accordingly, this result indicates that subpopulation of Ascl1^{+} progenitors in E14.5 ventral spinal cord gives rise to non-neural lineages. (A-C') Immunofluorescence image from P30 *Ascl1*$^{CreERT2/+}$*;R26R-stop-YFP* spinal cord after E14.5 tamoxifen administration. (B-B') 3D rendering images of YFP^{+} ependymal cells showing their unique morphology with round endfeet. (C-C'') Confocal images showing that YFP^{+} cells colabel with Sox2 (red) weakly and nuclei counterstaining marker TO-PRO-3 (blue).

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