2007

A Map of Transcription Factor Expression in Neuronal Precursors of the Chicken and Mouse Embryonic Cerebellum

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A map of transcription factor expression in neuronal precursors of the chicken and mouse embryonic cerebellum

A Thesis Presented to the Faculty of

The Rockefeller University

In Partial Fulfillment of the Requirements for

the degree of Doctor of Philosophy

by

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June 2007

A map of transcription factor expression in neuronal precursors of the chicken and mouse embryonic cerebellum

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The Rockefeller University 2007

The cerebellum is comprised of an overlying cortical structure and a basal set of cerebellar nuclei (CN). The cerebellar cortex contains two principal neurons, the Purkinje cell (PC) and the granule cell (GC), whereas the CN are formed by an heterogeneous array of cerebellar nuclei neurons. Although granule cell development is well-studied, the specification and early migratory routes of cerebellar nuclei neurons and Purkinje cells are not well understood. To map neural progenitor identity, we analyzed the expression of various members of the TALE, LIM and bHLH transcription factor families in the avian and murine cerebellar anlagen. We show here that the combinatorial expression of transcription factors Irx3, Meis1, Meis2, and Lhx2/9 defines neurons of the CN, and that the combinatorial expression of Lhx1 and Lhx5 defines Purkinje cells, as these distinct neural classes exit the cell cycle. We also show that Meis1 is a new marker for granule cell progenitors. Remarkably, the onset of transcription factor expression follows a precise temporal sequence, in concert with the emergence

of these three classes of cerebellar neurons. In addition, this study provides direct visualization of precursors of both the CN (Irx3^{+}, Meis1/2^{+}) and PC (Lhx1/5^{+}) moving along RC2/BLBP^{+} radial glial fibers in their transit from the ventricular zone (VZ) to the differentiating mantle zone. As cerebellar histogenesis proceeds, granule cell progenitors (Meis1^{+}/Math1^{+}) form the external granule layer and cerebellar nuclei neurons (Irx3^{+}/Meis1^{+}/Meis2^{+}) migrate circumferentially off the surface to settle as distinct nuclei underneath the emerging cerebellar cortex at early postnatal development. Thus, this transcription factor profile identifies the precursors of cerebellar nuclei neurons, Purkinje cells and granule cells from embryonic neurogenesis throughout postnatal stages, and provides a detailed spatiotemporal map of the morphogenetic events that form the cerebellar nuclei and the overlying cerebellar cortex.

Este estudio se lo dedico a mi maravillosa hija Simone

A mi linda familia

Angelica (mama), Marcelo (papa) y Darma (hermana)

y a Lolita

Acknowledgements

I am grateful to Tom Jessell and Arthur Buchberg for generously providing antibodies, and to Tom Jessell, Qiufu Ma, and Miguel Torres for *in situ* probes. I thank to Drs. Shiaoching Gong, Chen Zheng and Martin Doughty from the Gensat Project (Nat Heintz and Mary E. Hatten, Co-PIs), for the generation of the BAC mice used in this study. I wish to express profound thanks to Dr. Cori Bargmann, Dr. Ali Brivanlou, Dr. Nat Heintz, Dr. Tom Jessell and Dr. Carol Mason for helpful advice and discussions. I thank the National Scholarship Program from Chile for awarding me a Presidential fellowship. I want to gratefully acknowledge to all members of the Hatten lab, an extremely nice team that provide me continuous support and scientific interaction.

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Chapter 1

Introduction

1.1 The cerebellum: functions, evolution and circuitry

The cerebellum is a critical region of the vertebrate central nervous system (CNS) implicated in motor learning, motor coordination, balance, sensory discrimination, and cognitive processing (Boyden et al., 2004; Eccles, 1967; Fiez, 1996; Gao et al., 1996; Ito, 2002; Lisberger, 1998; Llinas and Welsh, 1993). The structure of the cerebellum is characterized by a set of cerebellar nuclei and a trilaminar cerebellar cortex (Fig. 1-1). The cerebellar cortex is a remarkable lattice with a single output neuron, the Purkinje cell and a principal interneuron, the granule cell (Jansen and Brodal, 1954; Larsell, 1967; Llinas and Welsh, 1993; Palay and Chan-Palay, 1974a; Ramon y Cajal, 1911). While the Purkinje cell receives a single class of inputs, climbing fibers, the granule cells receive mossy fiber inputs from numerous motor and sensory areas (Eccles, 1967). Although different regions of the cerebellum receive different inputs and project to different areas, the uniformity of the cellular structure of the cortex has suggested that different areas or modules process information in a similar manner (Boyden et al, 2004). Recent work showed that multiple plasticity mechanisms may contribute to cerebellar-dependent motor learning.

These multiple plasticity mechanisms include long-term depression (LTD) at the parallel fibre to Purkinje cell synapse (Ito, 2005), presynaptic and postsynaptic parallel fibre long-term potentiation (LTP) (Lev-Ram, et al., 2002; Linden and Ahn, 1999; Salin, et al., 1996), climbing fibre LTD (Hansel and Linden, 2000), LTP of inhibitory interneuron-Purkinje cell (PC) synapse (Jorntell and Ekerot, 2002; Jorntell and Ekerot, 2003; Khodakhah and Armstrong, 1997) and long-term, synaptic and non-synaptic changes in neurons of the cerebellar and vestibular nuclei (Aizenman and Linden, 2000). The existence of multiple plasticity mechanisms may provide the basis to learn and store motor movements over different time scales, to allow bidirectional movements and to provide directionality of movements (Boyden et al., 2004).

Afferent pathways project to Purkinje cells and granule cells from the thalamus, brainstem and spinal cord via the pontine nucleus and the olivary nucleus (Chedotal and Sotelo, 1992; Llinas et al., 2002; Nieuwenhuys et al., 1998; Ramon y Cajal, 1911; Sotelo and Chedotal, 2005). The Purkinje cell receives a single extracerebellar input from the climbing fibers, whereas the granule cells receive mossy fiber inputs from numerous motor and sensory areas (Eccles, 1967; Ito, 1984; Mason and Gregory, 1984; Mason et al., 1990; Mason et al., 1997;

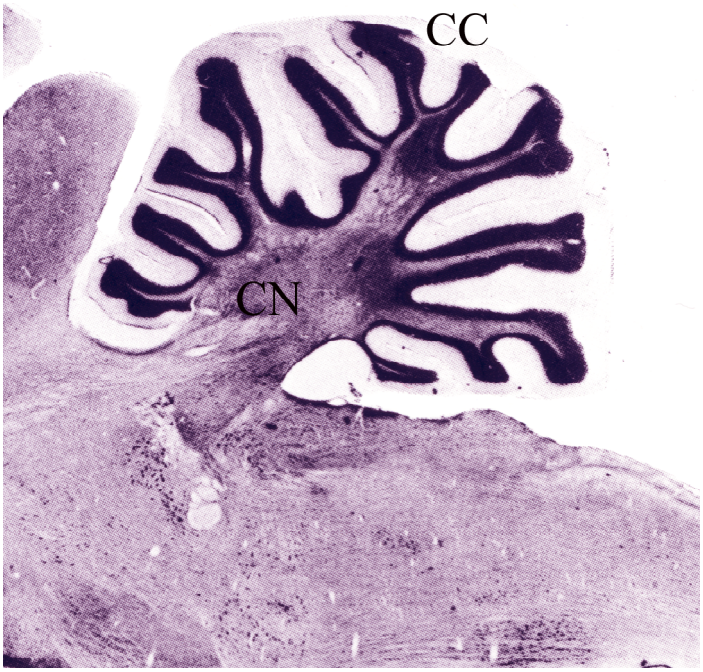


Figure 1-1 Cytoarchitecture of the adult cerebellum.

The architecture of the adult cerebellum consists of a trilaminar cerebellar cortex and a set of three cerebellar nuclei. The circuitry of the cerebellar cortex has a single output neuron, the Purkinje cell and a principal interneuron, the granule cell. Purkinje cells form several hundred thousand synapses with granule cell axons, which activity is mediated by as many as seven classes of interneurons. Cortical output occurs via Purkinje cell connections with neurons of the cerebellar nuclei, which project efferent fibers to the thalamus, brainstem and spinal cord. CC: cerebral cortex, CN: cerebellar nuclei.

Nieuwenhuys et al., 1998). Recently, it has been shown that granule cells, depending on their stage of development, can influence the growth of mossy fibers *in vitro* and *in vivo* (Manzini et al., 2006). Indeed, immature granule cells (in their cell progenitor state) inhibit the growth of mossy fibers, whereas that mature granule cells promote both the outgrowth of mossy fibers and the establishment of synaptic contacts (Manzini et al., 2006). Within the cerebellum, Purkinje cells receive several hundred thousand excitatory synapses from granule cell axons, an inhibitory inputs from seven classes of interneurons (Larsell, 1967; Mason et al., 1997; Morin et al., 2001; Mugnaini and Floris, 1994; Palay and Chan-Palay, 1974a; Ramon y Cajal, 1911; Voogd and Glickstein, 1998). Cortical output occurs via Purkinje cell connections with neurons of the cerebellar nuclei, which in turn project efferent fibers to the thalamus, brainstem and spinal cord (Cholley et al., 1989; Teune et al., 2000).

1.2 Patterning of the cerebellar territory along the rostrocaudal axis

During embryogenesis, the vertebrate hindbrain is transiently segmented into neural segments termed rhombomeres (r1-r8) between embryonic days E8.5-9.5 in the mouse, and stages 9-12 in the chick (Fraser et al., 1990; Keynes and Lumsden, 1990; Niederreither et al.,

2000). The boundaries of the rhombomeres align with the borders of expression of specific Hox genes (Wilkinson et al., 1989). The cerebellar territory arises from the dorsal rhombomere 1 (r1) at the mesencephalic/metencephalic border (Bally-Cuif et al., 1995; Liu and Joyner, 2001; Wingate and Hatten, 1999). Inductive factors derived from the isthmus at this interface organize the allocation of cell fates within the cerebellar anlage. Indeed, an isthmic gene regulatory network involving secreted proteins of the Wnt and Fgf superfamilies and the homeobox genes En1, Pax2/5/8, Otx2 and Gbx2 directs the formation and patterning of the anterior hindbrain, region that gives rise to the cerebellum (Danielian and McMahon, 1996; Irving and Mason, 2000; Martinez-Barbera et al., 2001; Wassarman et al., 1997; Wassef et al., 1993; Ye et al., 2001). The Fgf8 inductive factor controls a cascade of regulatory genes necessary for proper development of the mes/met organizer. Among them, Otx2 (expressed anteriorly) and Gbx2 (expressed posteriorly) establish reciprocal negative interactions that results in an $Otx2^{-}$ domain in the anterior hindbrain (r1-r3). The cerebellum, thus, arises from a territory that excludes Otx2 expression anteriorly and Hoxa2 expression posteriorly (r2) (Wingate and Hatten, 1999) (Fig. 1-2). The isthmic gene regulatory network is evolutionary conserved from fish to frogs to birds and mammals suggesting that the definition of the cerebellar territory along the anteroposterior axis of the

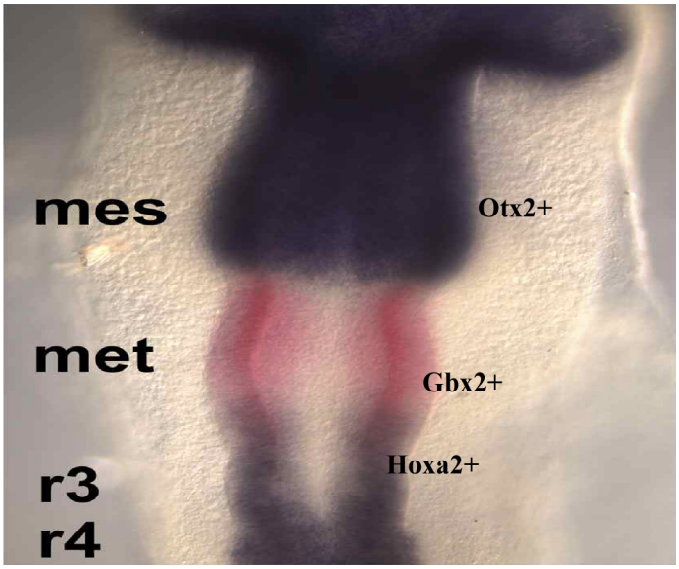


Figure 1-2 Definition of the cerebellar territory along the anteroposterior axis

Dorsal view of the mesencephalon/metencephalon (mes/met) region of an E2 (Stage 10) embryo triple-labeled by in situ hybridization for Otx2 (blue), Hoxa2 (blue) and Gbx2 (red). Otx2 and Gbx2 establish reciprocal negative interactions that results in an Otx2- domain in the anterior hindbrain (r1-r3). The cerebellum arises from a territory that excludes Otx2 expression anteriorly and Hoxa2 expression posteriorly (r2) (Wingate and Hatten, 1999).

neural tube follows a core developmental program.

While work over the last fifteen years has provided considerable details on the role of inductive signaling pathways and cell-intrinsic transcription factors during early patterning of the cerebellar territory, much less is known about the molecular basis of allocation of cell fate in the cerebellar anlage from the time when embryonic neurogenesis begins through postnatal development.

1.3 Cerebellar neurogenesis

The adult cerebellum consists of five major neural cell types including granule cells, Purkinje cells, stellate/basket cells, Golgi cells and cerebellar nuclei neurons (Goldowitz and Hamre, 1998; Maricich and Herrup, 1999). These cell types have highly been conserved through vertebrate evolution and their timetable of neurogenesis have been described in chick and mouse (Feirabend et al., 1985; Miale and Sidman, 1961; Voogd and Glickstein, 1998). The germinal neuroephitelium that gives rise to cerebellar cells is located in the roof of the IV ventricle, corresponding to the dorsolateral part of r1 (Hatten, 1999). Within this germinal neuroephitelium can be recognized two topographically segregated proliferative zones: the anterior rhombic lip (aRL) and the ventricular zone (VZ). In the embryonic mouse, cerebellar nuclei neurons appear at about E10, Purkinje cells start to be generated at E11 (Miale and

Sidman, 1961; Pierce, 1975; Voogd and Glickstein, 1998), while granule cells and Golgi cells are generated subsequently (Goldowitz and Hamre, 1998; Maricich and Herrup, 1999; Marti et al., 2001). The stellate/basket cells are apparently produced in postnatal stages of the mouse.

Upon leaving the germinal neuroephitelium, precursors of cerebellar nuclei neurons begin to differentiate and cluster into distinct cerebellar nuclei. During the formation of the nuclei, the Purkinje cells migrate out of the VZ to settle in the mantle zone as a thick plate of neurons. Beginning at E13, streams of granule cell progenitors move away from the aRL onto the surface of the anlage, beneath the pial surface, where they will form the external germinal layer, EGL (Harkmark, 1954; Hatten, 1999). After birth, Purkinje cells align into a monolayer and granule cell progenitors migrate inward, past Purkinje cells, to form the internal granule layer, IGL (Edmondson and Hatten, 1987; Rakic, 1971; Ramon y Cajal, 1889). These orchestrated cell movements set up the three layers characteristic of the adult cerebellar cortex, namely the molecular layer, the Purkinje cell layer, and the granule cell layer, (Hatten, 1999; Kuhar et al., 1993). In turn, the cerebellar nuclei neurons settle as distinct nuclei underneath the emerging cerebellar cortex.

1.4 Neural cell diversification in the vertebrate neural tube

During embryogenesis of the vertebrate CNS, diverse neural cell types are generated from progenitors cells located in defined positions within the ventricular zone of the neural tube. The process of neural cell diversification occurs as progenitor cells in active cell division in the ventricular zone give rise to postmitotic neurons which migrate outward through the germinal zone to settle in the differentiating mantle zone. How individual cell identities are ultimately assigned depends on the regulatory interaction over time between secreted local signals and intracellular transcription factors. The differential expression of transcription factors appears to be critical to define neuronal subtypes at distinct axial levels of the neural tube. In particular, a principle that emerged from a variety of studies focused on the histogenesis of the spinal cord, ventral midbrain and the cerebral cortex, is that the combinatorial action of homeodomain transcription factors underlies the generation of diverse neural cell types (Englund et al., 2005; Lee and Pfaff, 2003; Sanders et al., 2002).

In the developing spinal cord, distinct classes of postmitotic neurons (Lee and Jessell, 1999; Shirasaki and Pfaff, 2002) can be delineated by the combinatorial expression of transcription factors along the dorsoventral axis (Dasen et al., 2005; Gowan et al., 2001; Jessell, 2000; Moran-Rivard et al., 2001; Muller et al., 2002). The spinal cord is

subdivided into dorsal and ventral halves. In the dorsal half, six cell populations are defined by the differential expression of transcription factors, namely dI1: Lhx2/9, Brn3a; dI2: Lhx1/5, Brn3a, Foxd3; dI3: Isl1/2, Brn3a; dI4: Lbx1, Lhx1/5; dI5: Lbx1, Lmx1b, Brn3a; dI6: Lbx1, Lhx1/5, Dbx1, Dbx2 (Fig. 1-3).

In turn, the ventral half of the spinal cord is also defined by specific homeodomain protein profiles. The most dorsal cell population, the V0 interneurons are labeled by Evx1/2, Dbx1, Dbx2. V1 interneurons express En1 and Dbx2. V2 interneurons express Lhx3. Motorneurons express Isl1. V3 interneurons, which arise ventrally to motor neurons, express the bHLH transcription factor Sim1.

1.5 Transcriptional mechanisms implicated in cell fate allocation

The study of the transcriptional mechanisms required for neural cell fate specification has recently began to give important insights. The establishment of cross-regulatory interactions between transcription factors that localize to adjacent patterning domains is essential for allocation of regional cell fate. In the developing hindbrain the assignment of cell identity along the anteroposterior axis involves the establishment of sharp expression boundaries of Hox transcription factors at interfaces between rhombomeres. The maintenance of segments with distinct gene expression profiles depend upon auto-

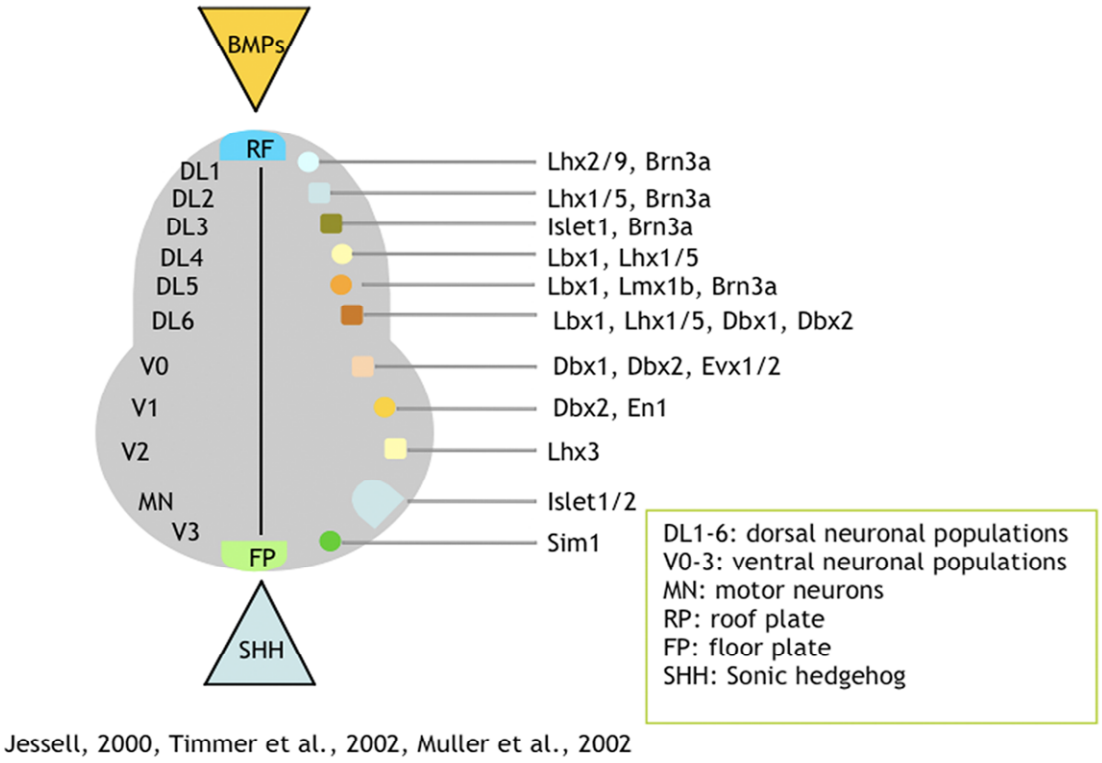


Figure 1-3 Combinatorial expression of transcription factors in the developing spinal cord. Distinct classes of postmitotic neurons can be defined by the expression of unique combinations of transcription factors along the dorsoventral axis. Shh produced in the floor plate (FP) induces ventral neural populations (V3-V0 and MN, motorneurons). BMPs expressed in the roof plate (RF) induce the most dorsal neural populations (DL1-3) which do not express Lbx1.

regulatory and cross-regulatory interactions between Hox transcription factors. First, retinoid signals derived from the adjacent paraxial mesoderm induce a first phase of Hoxb4 expression by activating a cis-regulatory element within the Hoxb4 gene. Then, Hoxb4 expression is maintained over time by both a positive autoregulatory feedback and Hox gene cross-regulatory interactions (Edlund and Jessell, 1999; Gould et al., 1997). Transcriptional autoactivation requires extrinsic signals to activate early response transcription factors which, in turn, initiate the expression of a cascade of transcription factors downstream. Transcriptional repression is an additional mechanism that explains how allocation of cell fate is regulated by transcription factors with non-overlapping expression patterns. In the developing spinal cord, motoneurons express various transcription factors, including the bHLH factors Ngn2, Olig1/2 and NeuroM and homeodomain proteins Nkx6.1, Nkx6.2, Pax6 and Hb9. As motoneuron cell progenitors exit the cell cycle, they begin to express Lhx3/4 and Isl1 and the homeodomain protein Hb9. A recent study showed that the specification of appropriate number of motoneurons involves both transcriptional activation and repression of the gene Hb9 (Lee and Pfaff, 2003). First, Hb9 expression is non-selectively initiated by general activators, e.g. E2F and Sp1, throughout the neural tube. Second, the cross-inhibitory actions of Irx3 and Nkx2.2 from adjacent regions

restrain Hb9 expression to motoneurons and prevent its expression in neighboring cells, defining a spatial domain where motoneuron differentiation proceeds. Third, a complex of LIM homeodomain proteins (Lhx3 and Isl1) and bHLH proteins (Ngn2, NeuroM and NeuroD) bind to an enhancer located upstream of the Hb9 gene for high-level activation of Hb9 transcription in postmitotic motoneurons. As the initiation and maintenance of Hb9 expression in differentiating motoneurons depend on the absence or inactivation of repressors from adjacent domains, this mechanism is called the derepression model (Lee and Pfaff, 2003). Thus, motoneuron specification involves the exclusion of Hb9 expression in the neighbouring non-motoneuron cells and the derepression of the Hb9 promoter in the motoneuron differentiation zone.

1.6 Role of transcription factors during histogenesis of the CNS

The retina

The mammalian retina comprises seven major classes of cell types (rod, cone, horizontal, amacrine, bipolar, ganglion, and Müller cells (Jeon et al., 1998; MacNeil and Masland, 1998; MacNeil et al., 1999). Cell lineage analyses have shown that during retinogenesis, each of these distinct cell types is generated from multipotent retinal

progenitor cells in an evolutionarily conserved birth order (Turner and Cepko, 1987; Turner et al., 1990). Ganglion cells are among the first cells to be produced, and bipolar cells are among the last. The retinal cells are organized into three cellular layers (outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL) and two synaptic layers (outer plexiform layer (OPL), the inner plexiform layer (IPL). A single retinal progenitor can give rise to all seven cell types, but a progenitor cell may also generate a subset of a single cell type. During development, retinal progenitor cell nuclei oscillate between the apical and basal surface as they progress through the cell cycle. Cells in S-phase are located near the basal surface of the outer neuroblastic layer (ONBL) and postmitotic cells are located near the apical surface of the ONBL. As development proceeds, a subset of postmitotic cells migrates to the inner neuroblastic layer (INBL) and differentiates into the major cell classes. Other subsets of postmitotic cells remain near the apical surface before settling in their final laminar position in the retina.

A number of genes show temporally restricted expression in retinal progenitor cells within the ONBL. For instance, *Lhx2* is weakly expressed in subsets of cells in the ONBL until P0, when it is transiently upregulated within the ONBL. *Math5* is expressed in subsets of mitotic

retinal progenitor cells, a gene that has been shown to be required for retinal ganglion cell development (Brown et al. 2001; Wang et al. 2001). Also, *Sox2, Lhx2,* and *Eya2* and others transcription factors as well as signal transduction components are expressed by subsets of presumptive retinal progenitor cells. Some transcription factors, including *Sox2, Sox4, Tbx2, Eya2* and *Mbtd1*, show differential expression in small subsets of cells within the ONBL at certain times during development. Many of these genes exhibit highly dynamic expression patterns. For example, *Pum1* and *Sox2* change their cellular expression patterns after a few days, whereas *Eya2* and *Pgrmc2* are expressed transiently.

The retina consist of up to 70% of rod photoreceptors which makes this cell type especially attractive for the analysis of selective gene expression (Jeon et al. 1998*;* Young et al. 1985). Transcription factors known to be involved in cell fate specification sometimes show broad expression in mitotic progenitor cells and persistent expression in mature cell types (Belecky-Adams et al. 1997*;* Liu et al. 1994; Livesey and Cepko 2001). The *Yboxbp4* transcription factor is expressed in early ONBL from E16 on, with expression persisting in mature photoreceptors. A similar pattern is observed for *castor*, though this gene is expressed in a more restricted subset of cells in the ONBL at

E16, and for the orphan nuclear receptor *ERRβ,* though this gene is expressed weakly during embryogenesis and its expression is dramatic increased in an undefined subset of cells in the immature photoreceptor layer during the first postnatal week. Also, a number of transcription factors are selectively expressed in differentiating photoreceptors. The *Rax* homeodomain factor is expressed in mitotic progenitor cells in the ONBL but is extinguished at the end of mitosis. However, its expression transiently reappears in immature photoreceptors at P8. *PIAS3,* which encodes a SUMO lyase that regulates the expression of various transcription factors (Haider et al. 2001), is selectively expressed only in developing photoreceptors from E18 on but it is not expressed in the adult retina, a pattern that is similar to the one exhibited by *Crx*. *Crx* is expressed in the outer region of the neural retina, the prospective photoreceptor layer, from E12.5 on but at moderate levels. At postnatal stages, *Crx* also showed expression in the photoreceptor layer with peak expression at P6 but *Crx* expression is decreased at P9. Expression of *Crx* mRNA is expressed in mature photoreceptors in adults. In contrast to these patterns, *Nrl* and *NR2E3* are not expressed prenatally, and then have a peak of their expression around P6.

Recent studies in various model organisms such as *Drosophila* neuroblast specification and the specification of neural-crest-derived cells (Anderson 1999; Isshiki et al. 2001; Pearson and Doe 2003) have

shown the role of temporal changes in gene expression in the specification of neural cells. In the retina, cell-fate specification is thought to occur through changes in competence by retinal cell progenitors. It has been proposed that multipotent retinal progenitor cells undergo unidirectional transitions in competence to generate each retinal cell type in the proper birth order (Cepko et al., 1996). According to this model mitotic progenitor cells would exhibit both temporal changes in gene expression across broad sets of retinal progenitors, and expression of selected genes in specific subsets of progenitor cells at a given time. For example, early-stage retinal progenitor cells are competent to give rise to early-born cell types such as ganglion cells, and late-stage retinal progenitor cells are competent to give rise to late-born cell types such as bipolar cells. In addition to intrinsically changing retinal progenitor cell competence, extracellular (extrinsic) factors of the developing retina also influences retinogenesis (Belliveau and Cepko, 1999; Belliveau et al., 2000; Wang et al., 2002; Dakubo et al., 2003; Young and Cepko, 2004).

The spinal cord

In the developing spinal cord, the expression of homeodomain proteins define distinct cell populations along the dorsoventral axis. Lhx2 and Lhx9 are expressed by the two most dorsal cell types which are termed dI1A and dI1B neurons, respectively. Other dorsal cell populations are defined by Lhx1,5 (dI2 neurons) and Isl1 (dI3 neurons) expression (Gowan et al., 2001; Jessell, 2000; Muller et al., 2002; Muller et al., 2003).

In vitro studies showed that BMP4, BMP5, BMP7, DSL1 and activin A are necessary to induce dI1 neurons in spinal cord explant preparations (Liem et al., 1997). The generation of this cell population appears to be regulated by BMP signals derived from the roof plate (Liem et al., 1997). The roof plate is a transient signaling center on the dorsal midline of the neural tube that coordinates dorsal CNS development through the action of local peptide signals, primarily the bone morphogenic proteins (BMPs) and the Wingless-related genes (Wnts). The role of the roof plate has become evident through studies of mutations of genes in these gene families, and through several spontaneously occurring mouse mutants, including *dreherJ* (*drJ*), all of which cause dorsal neural tube defects.

The positional cloning of the spontaneously occurring neurological mutant mouse *dreher* (*drJ/drJ*) demonstrated that an inactivating point mutation in the LIM homeodomain (HD) transcription factor *Lmx1a*, is responsible for the *drJ* phenotype (Millonig et al., 2000). As the neural tube closes, *Lmx1a* expression is restricted to the roof plate along the neuraxis during CNS development. Phenotypic analyses demonstrated that the roof plate is almost missing in the *dreher* mouse. The development of the dorsal neural tube is then affected with a reduction in the number of cells in the dorsal horn of the spinal cord. In *drJ/drJ*, although non-functional *Lmx1a* is correctly expressed at E8.5–E9.5, its expression is lost in the spinal cord roof plate by E10.5. Coincident with the loss of *Lmx1a* expression, *Bmp* expression fails, and the generation of the dorsal-most spinal cord neurons, the dI1 interneurons, is affected. In *drJ/drJ* embryos, defects in the migration of dI1 neurons is observed as well as in axon pathfinding.

Further evidence that support a role for the roof plate in patterning of the neural tube along the dorsoventral axis was provided by a study in which the genetic ablation of the roof plate was achieved by the conditional overexpression of a cellular toxin under the control of the *Gdf7* promoter. The findings showed that the most dorsal cell populations of the spinal cord, dI1 neurons, $Lhx2^{+}$/$Lhx9^{+}$ and dI3 neurons, $Isl1^{+}$ (dI3 neurons, previously defined D2) are missing in the

embryos lacking GDF7 (Lee et al., 2000). Recently, a study analyzed the phenotype of *Bmpr1a/Bmpr1b* double knockout mice in the developing spinal cord (Wine-Lee et al., 2004). The Bmp receptor double knockout mice showed a loss of dI1 neurons as evidenced by the lack of Math1 expression in progenitors and the absence of postmitotic markers Lhx2/9. A loss of dI2, Lhx1/5 and Foxd3 expressingneurons is also observed in the double knockout mice. As a consequence, dI3 and dI4 interneurons shift to a most dorsal position, adjacent to the roof plate. Taken together, these findings demonstrate that BMP signaling is required for the generation of the most dorsal interneurons of the developing spinal cord.

The cerebellum

At E9.5 in the mouse, the neural tube is formed as the neural plate folds elevate and coalesce, event that defines the future dorsal midline of the neural tube. After closure of the neural tube, the expanded rostral portion of the neural tube becomes subdivided into three vesicles: the forebrain, midbrain and hindbrain.

In the cerebellum, a structure derived from the alar plate (dorsal) of the r1, the rostral part of the hindbrain, cell proliferation begins at about E10. Granule cell progenitors within the aRL, a germinal neuroephitelium in close proximity to the r1's roof plate, begin to

express several transcription factors, including the zinc finger genes Zic1 and Zic2 and the bHLH gene Math1. The idea that granule cell specification can be induced by dorsal signals was tested in the mouse by Alder et al., (1999).

This work analyzed expression patterns of various members of the BMP family at the time when granule cell progenitors are specified. The authors found that Gdf7, Bmp6 and Bmp7 are expressed by dorsal midline cells adjacent to the nascent aRL. Bmp7 is expressed within roof plate cells, whereas both Bmp6 and Gdf7 are expressed in cells of the dorsal neuroephitelium adjacent to the roof plate. *In vitro* induction assays were designed to see whether BMP signals are able to initiate granule cell differentiation. Granule neuron progenitors were assessed by colocalization of En1/2, Math1, and Zic1, Zic2 transcription factors. Neural plate explants isolated from dorsal or ventral regions of E8.5 (6-10 somite stage) were grown for 72 hours *in vitro* before analysis. Treatment of ventral explants with BMP6, BMP7 or GDF7 induced expression of the dorsal markers Math1, Zic1 and Wnt3a, typical of granule cell progenitors. Importantly, this study demonstrated that BMP-treated ventral neural cells are able to form mature granule neurons when transplanted into the early postnatal cerebellum, suggesting that BMPs induce granule cell specification in the dorsal cerebellum.

A study focused on the dorsalizing defects of the spontaneous mutant *dreher* mice provided a genetic demonstration for the idea that the roof plate was involved in dorsal pattering of the cerebellum (Millonig et al., 2000). This study showed that the roof plate is missing at the level of the cerebellar territory in *dreher* mice as assessed by MafB expression. Whole-mount *in situ* hybridization with *Math1* also revealed that the aRL was significantly reduced in *drJ*/*drJ* embryos at E11.5 (Millonig et al., 2000). At E12.5, the choroid plexus, a roof plate derivative, is also missing. The phenotype of *dreher* mice was also analyzed at later embryonic stages. In *drJ/drJ* embryos, the choroid plexus, the aRL and granule cell progenitors were missing in many sections by E14.5, and consequently the size of the cerebellar anlage was reduced. Recent work further analyzed the role of the roof plate in cerebellar development by using gain- and loss-of-function in mice (Chizhikov et al., 2006). The authors used a set of transcription factors, including Math1, Lmx1a, Lhx1/5, Lhx2/9 and Ptf1a to define distinct cerebellar cell populations at stages E10.5 and E12.5. They defined four classes of cells, which they termed c1-c4. The c1 cell population (cells derived from the aRL), is defined by Math1 expression. As c1 cells moved out of the aRL, Lhx2/9 is turned on. In turn, Lhx1/5 is expressed in c2 cells (Purkinje cells), Lmx1a is expressed in c3 cells (cerebellar nuclei neurons), and Lhx1/5 is expressed in c4 cells (an

unknown cell population locasted more ventrally) (Chizhikov et al., 2006). Using these markers to track the different cell populations, the authors showed that signals derived from the roof plate are necessary and sufficient for the generation of c1 cells. However, the specification of c2 and c3 cell population proceeds normally when the roof plate is ablated in transgenic mice engineered to kill the roof plate cells that normally express *Gdf*, a gene implicated in roof plate development. The number of c2 and c3 cells are reduced in roof plate-ablated mice (Chizhikov et al., 2006). These data demonstrated that, as is seen in the spinal cord, the roof plate is required for dorsal patterning within the cerebellum.

An additional study addressed the role of inductive signals, derived from the dorsal midline of the r1, in the specification of cerebellar cell classes (Qin et al., 2006). The authors examined *Bmpr1a;Bmpr1b* double knock-out mice to demonstrate genetically that signaling via type I BMP receptors is required for the specification and differentiation of granule cells and that the receptor genes *Bmpr1a* and *Bmpr1b* are functionally redundant in this function. They observed severe cerebellar patterning defects, resulting in smaller cerebella that are devoid of foliation. In mutants containing either single BMP receptor gene mutation alone, cerebellar histogenesis proceeds normally, showing functional redundancy of type I BMP receptors during

cerebellar development. Loss of BMP signaling in double mutant animals causes a dramatic reduction in the number of cerebellar granule cells; many of those that remain are in ectopic locations. Molecular markers of granule cell specification, including *Math1* and *Zic1*, are drastically downregulated. In addition, Purkinje cells are disorganized and ectopically located, but they appear to be correctly specified. Consistent with the interpretation that granule cells alone are affected, phosphorylated Smad1/5/8 is immunolocalized predominantly to granule cell precursors and is not detected in Purkinje cell precursors. This study demonstrates that BMP signaling plays a crucial role in the specification of granule cells during cerebellar development.

Recent studies have also highlighted the importance of cell-intrinsic mechanisms in neural cell fate specification and differentiation in the embryonic cerebellum. The gene *Ptf1a*, which encode a transcription factor of the bHLH family, was identified as a critical factor involved in the generation of GABAergic neurons in the developing cerebellum (Hoshino et al., 2005). Ptf1a expression is restricted to the VZ. Cell lineage analyses showed that there are two subsets of neural progenitors in the VZ: (i) neural progenitors that express Ptf1a and generate GABAergic neurons, including Purkinje cells, Golgi cells, basket/stellate cells and small cerebellar nuclei neurons, and (ii) cell

progenitors that do not express Ptf1a and which are involved in the production of glutamatergic neurons in the cerebellar nuclei. In mouse, loss of *Ptf1a* results in a defective generation of GABAergic neurons. Also, the EGL is secondarily lost. As a consequence adult mutants lack the entire cerebellar cortex. This cerebellar phenotype motivated the authors to name this mutant "*cerebelles*" (*cbll*) (Hoshino et al., 2005).

The involvement of the transcription factor Pax6 in neural cell allocation has been established in the cerebellar system (Landsberg et al., 2005). Using a genetic fate mapping approach, this study shows that the posterior rhombic lip (pRL), defined by *Wnt1* expression, is organized in distinct spatial domains along the dorso-ventral axis which are well-defined molecularly. *Lmx1a* and *Gdf7* is expressed in the most dorsal domain which is occupied by progenitors of the choroid plexus. *Math1* is expressed in progenitors that give rise to mossy fiber nuclei within the brainstem. The authors showed that the primordium of the climbing fibers derives, in part, from the *Wnt1* territory and that maps ventral to the *Math1*$^{+}$ domain. *Ngn1* is expressed in neural progenitors of the climbing fiber nucleus. Interestingly, *Pax6* appears to regulate neural cell allocation in the pRL. Analyses of *Pax6* null mice show that loss of *Pax6* results in an expansion of the *Ngn1* domain and a reduction of the *Math1* domain. Furthermore, *Pax6* mutants have an

enlarged climbing fiber nucleus and reduced mossy fiber nuclei (PGN, RTN, LRN and ECN). This study provides direct evidence for a role of *Pax6* in neural cell allocation within the pRL. Also, these findings suggest that *Pax6* regulates the production of the two major afferences of the precerebellar system (Landsberg et al., 2005).

In this thesis, I aimed to understand how are cerebellar nuclei neurons, Purkinje cells and granule cells allocated to nuclear and cortical fates in the embryonic cerebellum. I showed that the differential expression of unique combinations of transcription factors define the major cerebellar cell classes in the developing cerebellum. A set of markers for cerebellar nuclei neurons, Purkinje cells and granule cell progenitors was identified from the time that neurogenesis begins in the avian and murine cerebella. The fact that gene expression of various transcription factors persisted over time enabled to delineate the migratory pathways that progenitors followed from their origin in the cerebellar germinal zones to their final positions in the mantle zone. Direct evidence is provided to show that cerebellar nuclei neurons and Purkinje cells migrate out of the VZ in close apposition to radial glial fibers. The concerted cell movements that drive the assembly of neurons into the cerebellar nuclei and overlying cerebellar cortex were similar in both chicken and mouse model organisms. Taken together, these studies suggest that the differential expression of transcription

factors have a critical role in allocation of neural progenitors to the distinct nuclear and cortical structures of the cerebellum.

Chapter 2

A map of transcription factor expression in the developing cerebellum

Introduction

Given the central role of transcription factors in allocation of cell fate in the CNS, the present study focuses on transcription factor expression in cerebellar nuclei neurons, Purkinje cells and granule cells from the beginning of embryonic neurogenesis through postnatal stages.

Determining transcription factor expression profiles may provide a key framework to understand how neural cell diversity is generated during histogenesis of the cerebellum. Despite classical work established the anatomy and physiology of the postnatal cerebellum, the absence of molecular markers for precursors of the cerebellar nuclei and Purkinje cells has precluded studies on the developmental programs that control the allocation of these neurons to the cerebellar nuclei and cerebellar cortex, critical for the formation of the cerebellar circuitry. In this study, we mapped spatiotemporal patterns of transcription factor expression during cerebellar development to reconstruct, at cellular resolution, the

time of origin and patterns of migration of deep neurons, Purkinje cells and granule cells.

2.1 Analysis of transcription factor expression in the mouse and chick cerebellar anlagen

The expression of various transcription factor families was studied by combining immunohistochemistry, *in situ* hybridization and analyses of BAC transgenic mice. The transcription factors studied in this work include the TALE (Irx1, Irx2, Irx3, Meis1 and Meis2), LIM (Lhx1/5, Lhx2/9 and Lhx3), Paired-box (Pax2, Pax3 and Pax6), Even-skipped (Evx1), Zic (Zic1/3, Zic2), and bHLH (Math1, NeuroD, Mash1, Ngn1, Ngn2) proteins (Table 2-1).

2.2 Definition of postmitotic territories within the cerebellar anlage

To link gene expression and neural identity, we correlated cytoarchitecture and gene expression patterns. At the beginning of neuronal differentiation, the postmitotic cell zones of the mouse cerebellum can be delineated by the expression of class III beta-tubulin (ß-Tub). At E10.5, ß-Tub expression was restricted to cells occupying the surface of the anlage (Fig. 2-1A). This differentiating zone consists of prospective cerebellar nuclei neurons (Pierce, 1975). At E11.5, ß-Tub expression persisted in the surface but was also detected in precursors

of cortical cells in the nascent intermediate zone, just above the VZ (Fig. 2-1B). At E12.5, increased numbers of differentiating cells distribute in both differentiating zones (Fig. 2-1C) (Yuasa et al., 1991). Thus, the expression of ß-Tub defined the emergence of the two early differentiation zones of the anlage and showed that the first postmitotic neurons to appear during cerebellar histogenesis populate the dorsal surface of the anlage. By E12.5, Nissl staining reveals that the germinal neuroepithelium of the cerebellum is topographically subdivided into the VZ and the aRL (Fig. 2-1D) (Hanaway, 1967; Wingate and Hatten, 1999). The histology of the mouse cerebellum has a similar organization to the one exhibited by the rat cerebellum (Altman and Bayer, 1985a). Differences in the nomenclature of various embryonic zones in the chicken, mouse and rat systems have complicated comparisons between the three. In particular, the superficial zone where cerebellar nuclei neurons begin to differentiate in the anlage has been named either as roof nuclei or nuclear transitory zone (NTZ) (Altman and Bayer, 1985a; Miale and Sidman, 1961; Pierce, 1975). Also, the terms Purkinje cell plate and cortical transitory zone (CTZ) have been used to refer to the early differentiating zone of Purkinje (Altman and Bayer, 1985a; Goffinet, 1983). We have used a nomenclature that distinguishes two major regions within the embryonic cerebellum,

Table 2-1 A list of the transcription factors analyzed in this work

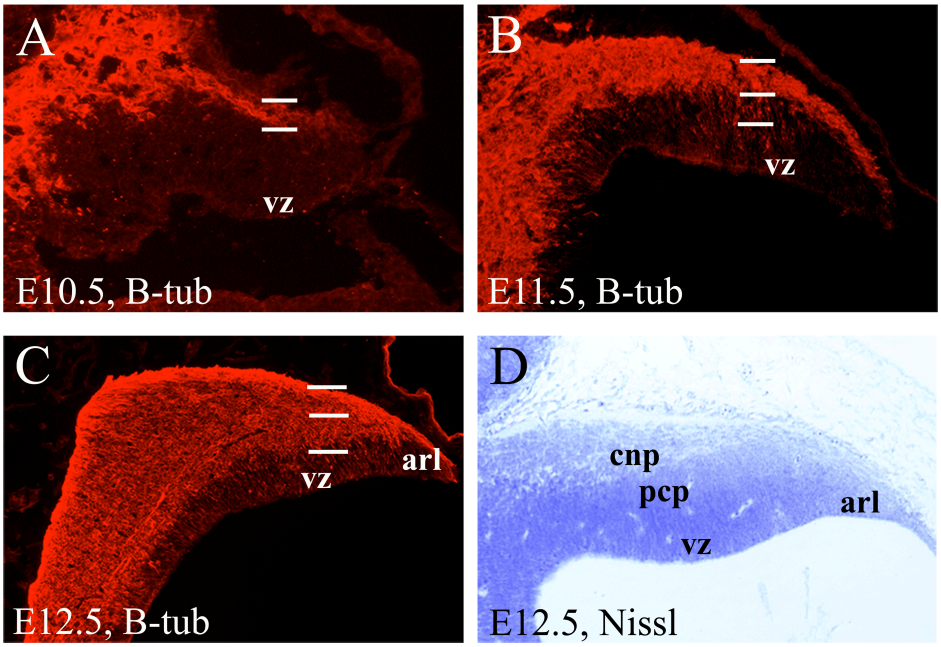
The expression of transcription factors was analyzed from stages Embryonic 10, E10 to Postnatal 7, P7 in mice, and from stages e4 (HH 23) to e8 (HH34) in chickens. High to low gene expression levels were assessed in the germinal ventricular zone, the anterior rhombic lip and the postmitotic differentiating zones. Abbreviations: + to +++: low to high expression. -: no expression.

Table 2-1

Gene	Ventricular Zone	Postmitotic Zone
Math1	+ (rhombic lip)	+++, EGL
Mash1	+	-
Neurogenin2	+	++, subventricular zone
Neurogenin1	+	++, subventricular zone
NeuroD	-	+++
Lhx1	-	+++
Lhx5	-	+++
Lhx3	-	-
Lhx2	-	+++
Lhx9	-	+++
Meis1	+++	+++
Meis2	-	+++
Irx3	+++	+++
Irx1	+++	++
Irx2	+++	++
Zic1,3	+++	+
Pax2	-	+++

Figure 2-1 Histological organization of the embryonic cerebellum at the beginning of neurogenesis.

(A) Sagittal section of the E10.5 mouse cerebellum labeled with beta-tubulin (ß-Tub) antibody. ß-Tub is expressed first by differentiating neurons positioned in the surface of cerebellar anlage. This superficial zone is populated by prospective cerebellar nuclei (CN) neurons. (B) A sagittal section of the E11.5 mouse cerebellum labeled with ß-Tub shows the emergence of a second differentiating zone, between the ventricular zone (VZ) and the surface. This zone is populated by Purkinje cells and interneuron cell precursors. (C) Sagittal section of the E12.5 mouse cerebellum. ß-Tub expression shows the two differentiating zones of the cerebellum. (D) Nissl staining of an E12.5 sagittal section shows that the cerebellar germinal proliferative zone is subdivided into two, the VZ and the anterior rhombic lip (aRL). As cerebellar nuclei precursors occupy the superficial zone, Purkinje cells position in the intermediate zone. The granule cell progenitors occupy the aRL.


A
vz
E10.5, B-tub
B
vz
E11.5, B-tub
C
vz
arl
E12.5, B-tub
D
cnp
pcp
vz
arl
E12.5, Nissl

namely the ventricular zone and the mantle zone (Yuasa et al., 1991). The ventricular zone is populated by proliferating cell precursors whereas the mantle zone consists of differentiating cells. Hence, cerebellar nuclei neurons differentiating transiently underneath the pia matter form the superficial mantle zone (that corresponds to Altman and Bayer's NTZ), and Purkinje cells that differentiate just above the VZ form the intermediate mantle zone (corresponding to the Altman and Bayer's CTZ).

Chapter 3

A map of transcription factor expression for precursors of the cerebellar nuclei

Introduction

Classical [3H]-thymidine studies have provided evidence that cerebellar nuclei neurons are generated by E10 in the mouse; cerebellar nuclei neurons are thought to be the first cerebellar neurons to appear in the anlage (Miale and Sidman, 1961; Pierce, 1975). To address the question of how cell progenitors are allocated to the cerebellar nuclei, we examined the expression of transcription factors Irx3, Meis1, Meis2, and Lhx2/9 (Liem et al., 1997; Toresson et al., 2000; William et al., 2003). The assignment of nuclear neuron identity was assessed by evaluating several criteria, including time of birth, timing of neuronal differentiation, axonal projections, and cell positions over time (see review, (Shirasaki and Pfaff, 2002)).

Results

3.1 Irx3, Meis1, Meis2 and Lhx2/9 are expressed by cerebellar nuclei neurons in the developing cerebellum

To identify molecular markers for cerebellar nuclei neurons we assayed gene expression at multiple stages of cerebellar development. At E10.25, the expression of Irx3, Meis1, Meis2 was prominent in postmitotic neurons distributed uniformly in the surface of the mantle zone, a region composed of neurons undergoing differentiation (Fig. 3-1 A,B). In turn, Lhx2/9 expression commenced in a few postmitotic cells scattered in this zone (Fig. 3-2 A, asterisks). Within neural cell progenitors, Irx3 expression was restricted to the VZ, whereas Meis1 expression was detected both in the VZ and the aRL (Fig. 4-1 B and data not shown). Moreover, Irx3, Meis1 and Meis2 labeled migrating cells moving from the VZ to the surface of the mantle zone at E10.25, presumably cell precursors of the cerebellar nuclei as the EGL is not present at this age and the generation of the other cerebellar neurons have not begun yet (Fig. 3-1 B).

Previous birthdating studies provide evidence that cells born in this period are progenitors of the cerebellar nuclei (Pierce, 1975). To confirm the identity of this progenitor cell population at E12.5, we carried out BrdU labeling experiments in combination with immunolocalization of Meis2. Sagittal sections of E12.5 mouse embryos

pulsed with BrdU at E10.25 showed large numbers of BrdU labeled cells across the dorsal surface (Fig. 3-1 C). The majority of the $BrdU^+$ cells expressed Meis2, indicating that prospective cerebellar nuclei neurons born at E10.25 have migrated outward into the superficial mantle zone of the E12.5 anlage.

By E12.5, increased numbers of postmitotic cells settle in the superficial mantle zone and a second postmitotic region positioned between the VZ and the surface emerged, the intermediate mantle zone (Fig. 2-1 C,E). The expression of Irx3, Meis2 and Lhx2/9 persisted in cells located in the surface (Fig. 3-1 C; Fig. 3-2 B). In contrast, Meis1 was expressed by cell progenitors located both in the superficial mantle zone and the aRL (Fig 4-1 B). At E14.5, Irx3, Meis1, Meis2, and Lhx2/9 expression profiles reveal a mass of cells clustering in the anterior cerebellum (Fig. 3-1 D,E; Fig. 3-2 C; Fig. 4-1 D). Thus, the spatial and temporal patterns of expression of Irx3, Meis1, Meis2 and Lhx2/9 reveal prospective cerebellar nuclei neurons during the process of nuclei formation.

To examine whether individual cerebellar nuclei neurons expressed combinations of these transcription factors, we performed double immunostaining on tissue sections from *Irx3* BAC transgenic

Figure 3-1 The expression of Irx3, Meis1, Meis2, and Lhx2/Lhx9 defines the precursors of the cerebellar nuclei

Representative photos showing expression of Irx3, Meis1, Meis2, and Lhx2/Lhx9 in sagittal (A-F) and transverse (G, H) sections at various embryonic stages of the mouse cerebellum. (A) At E10.25, Meis2 is expressed by prospective nuclear neurons that have reached the superficial zone. (B) High power confocal picture show columns of $Irx3^+$ cells as they transit from the VZ to the surface. (C) Meis2 antibody staining of an E12.5 mouse embryo pulsed with BrdU at E10.25 shows that prospective nuclear neurons generated at E10.25 ($Meis2^+/BrdU^+$) occupy the surface by E12.5. At E14.5, Meis2 (D) and Meis1 (E) are expressed in nuclear neurons clustering in the rostral cerebellum. (F) Staining of E14.5 *Irx3* BAC transgenic mice with EGFP and Meis2 antibodies shows that nuclear neurons coexpress Irx3 and Meis2. (G) At E15.5, EGFP staining of an *Irx3* BAC transgenic mice reveals the process of nuclear formation. Nuclear neurons of the medial (fastigial) cerebellar nucleus project an $Irx3^+$ axonal tract that decussate within the cerebellar dorsal midline to reach the contralateral homologous nucleus. (H) At E15.5, *Irx3* BAC transgenic mice reveal nuclear neurons segregating into distinct nuclei within the mediolateral axis. cn: cerebellar nuclei, egl: external germinal layer.

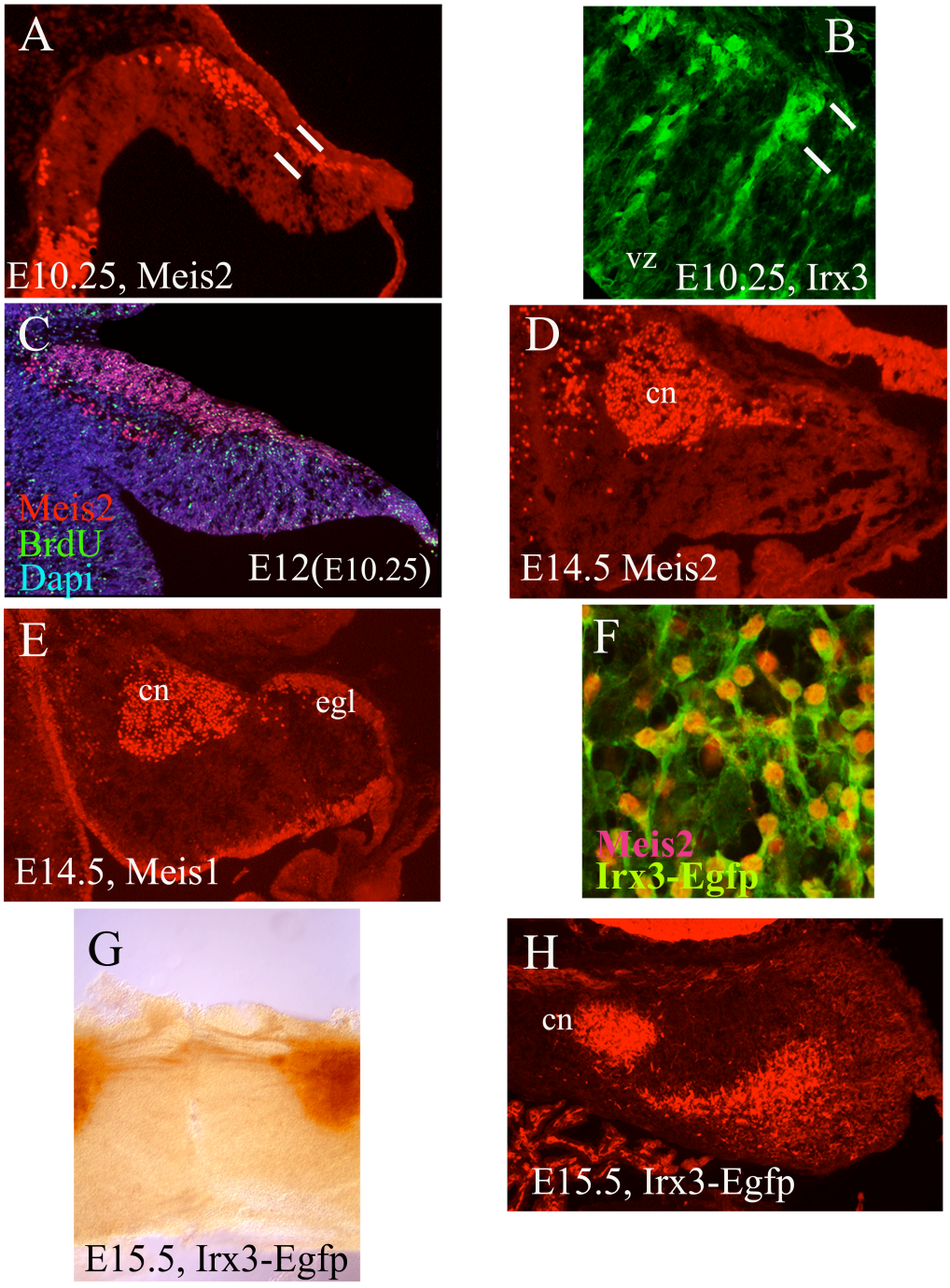
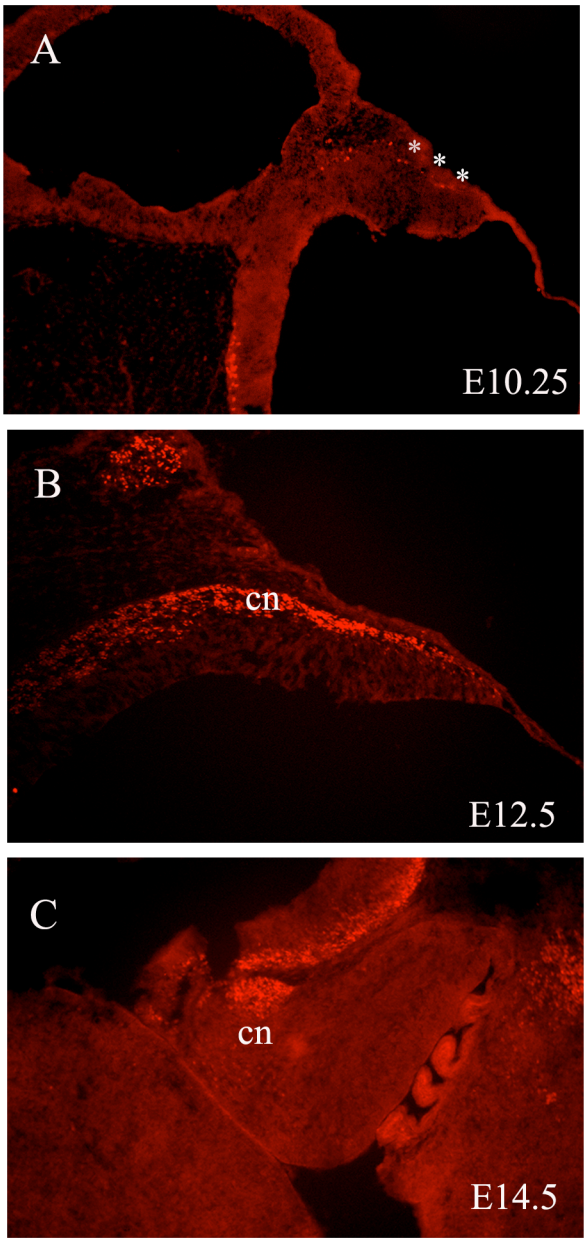

A
E10.25, Meis2
B
vz
E10.25, Irx3
C
Meis2
BrdU
Dapi
E12(E10.25)
D
cn
E14.5 Meis2
E
cn
egl
E14.5, Meis1
F
Meis2
Irx3-Egfp
G
E15.5, Irx3-Egfp
H
cn
E15.5, Irx3-Egfp

Figure 3-2 Lhx2/9 is expressed by cerebellar nuclei precursors

Sagittal sections of E10.25 (A), E12.5 (B) and E14.5 (C) mouse embryos. (A) Expressio of Lhx2/9 begins at E10.25 in the embryonic cerebellum. A few postmitotic Lhx2/9 expressing cells are located in the surface of the anlage. (B) At E12.5, Lhx2/9 expression persisted in postmitotic cells located in the surface. (C) At 14.5, Lhx2/9 is expressed by cerebellar nuclei precursors clustering in the rostral cerebellum.

Lhx2/9



E14.5, Lhx2/9

mice. Immunostaining of E14.5 *Irx3* BAC transgenic mice, with antibodies specific to EGFP and Meis1, Meis2 revealed many Irx3/Meis2 and Irx3/Meis1 double-labeled cells within a cluster of neurons during nuclei formation, the emerging cerebellar nuclei in the anterior cerebellum (Fig. 3-1 F).

By E14.5, cerebellar nuclei neurons project axons to targets within the cerebellum and brainstem in rodents (Bourrat and Sotelo, 1986; Parenti et al., 2002). Strikingly, by E15.5, we observed that a well-defined $Irx3^{+}$ axonal tract emerging from the medial nuclear nucleus decussated within the cerebellar midline to target the contralateral homologous nucleus (Fig. 3-1 G,H). By E17, the paired medial cerebellar nuclei and the connecting $Irx3^{+}$ tract had descended to the ventral aspect of the anlage. Thus, Irx3, Meis1 and Meis2 mark all three sequential steps in the migratory pathway of cerebellar nuclei neurons -a radial migration along the glial scaffold to populate the surface, a tangential migration to the rostral cerebellum, and an inward migration to settle, as three discrete nuclei, in the depth of the cerebellum.

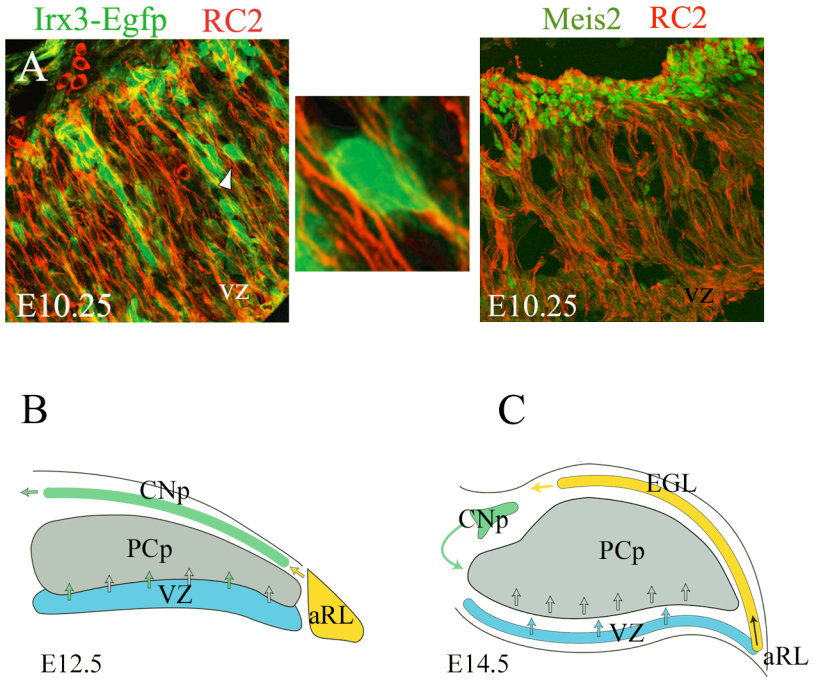
3.2 Radial, tangential and inward migration of precursors of the cerebellar nuclei during ontogeny

Since cell precursors of the cerebellar nuclei (Irx3$^+$, Meis1$^+$, Meis2$^+$) migrate radially from the VZ onto the dorsal surface by E10.25, we examined the possibility that this initial neuronal migration utilize a system of radial glial fibers. At E10.25, a thick network of radial glial fibers expressing the marker RC2 extended across the cerebellar wall (Misson et al., 1988). We found that virtually all RC2$^+$ cells co-expressed the brain lipid binding protein, BLBP, at this stage (Anthony et al., 2004; Feng et al., 1994). To examine the disposition of cerebellar nuclei neurons relative to the radial glial fibers, we stained transverse sections from *Irx3* BAC transgenic mice with antibodies against EGFP and RC2. High magnification confocal pictures of E10.25 transverse sections, revealed that Irx3$^+$ cerebellar nuclei neurons were juxtaposed to RC2$^+$ radial glial cells (Fig. 3-3 A, inset). As Irx3 was expressed by proliferative cells in the VZ and postmitotic cells located in the surface, these experiments allowed, for the first time, direct visualization of columns of prospective cerebellar nuclei neurons moving from the VZ to the surface along the radial glial fibers.

By E12.5 cerebellar nuclei neurons begin to shift the trajectory of their migration from a radial to a tangential orientation (Fig. 3-3 C, left

Figure 3-3 Three-step migratory pathway of nuclear neurons during nucleogenesis

(A) Left panel, a transverse section shows precursors of the cerebellar nuclei (Irx3$^+$, Meis2$^+$) migrating radially along radial glial fibers (RC2$^+$) as they transit from the VZ to the surface of the embryonic cerebellum. A high power confocal image shows an Irx3-EGFP BAC mouse labeled with RC2 and EGFP. Columns of Irx3+ cells migrate along radial glial fibers that span the entire wall of the anlage at E10.25. Inset shows an individual Irx3$^+$ migrating along a RC2$^+$ radial glia process. Right panel, Meis2 is expressed by prospective cerebellar nuclei precursors as they migrate along RC2$^+$ radial glia towards the surface at E10.25. (B) A schematic of the E12.5 mouse cerebellum shows cerebellar nuclei precursors (CNp, green, Irx3$^+$, Meis1$^+$, Meis2$^+$, Lhx2/9$^+$) migrating tangentially, in parallel to the pia matter, towards the rostral cerebellum. (C) A schematic of the E14.5 mouse cerebellum. CNp cells (green, Irx3$^+$, Meis1$^+$, Meis2$^+$, Lhx2/9$^+$) clustered in the rostral cerebellum undergo inward migration to eventually settle in the depth of the cerebellum at postnatal stages. Abbreviations: aRL: anterior rhombic lip; EGL: External germinal layer; PCp: Purkinje cell precursors; VZ: Ventricular zone.



Cerebellar nuclei precursors, CNp, express
Irx3, Meis1, Meis2 and Lhx2/9 at E12.5 and E14.5

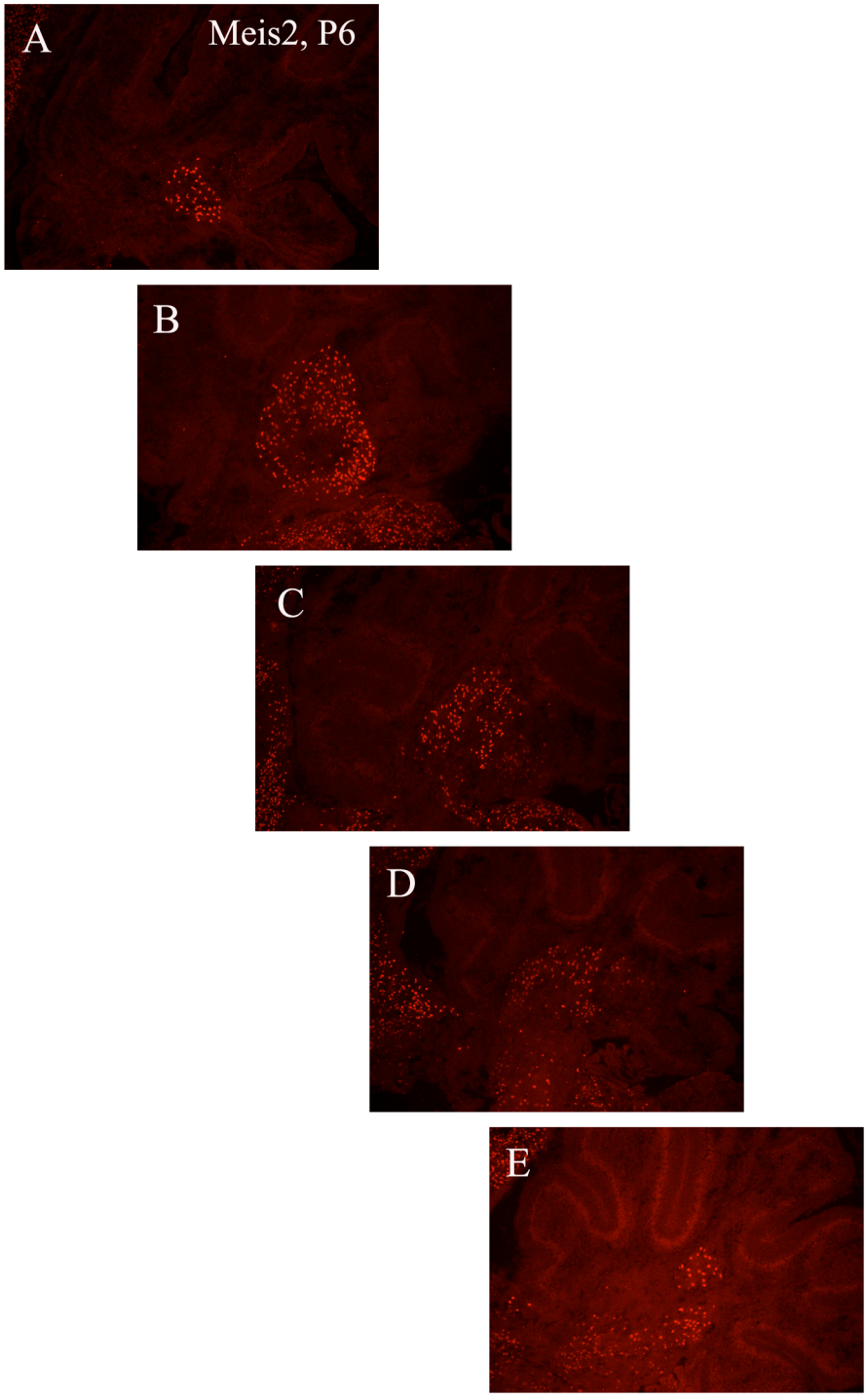
panel). Indeed, between E12.5 and E14.5, Irx3^{+} cells migrate tangentially along the dorsal surface toward the anterior aspect of the anlage. From E14.5 onward, cerebellar nuclei neurons migrate inwardly from the surface toward the depth of the cerebellum where they form three distinct nuclei (Fig. 3-3 C, right panel). Thus, the morphogenesis of the cerebellar nuclei involves three migratory phases, including an initial radial migration of cerebellar nuclei neurons along glia to pioneer the surface of the anlage, a second migratory phase consisting of cell movement in the tangential plane, parallel to the overlying pia matter, and a third phase, the inward migration of cerebellar nuclei neurons to settle underneath the developing cerebellar cortex.

3.3 Transcription factor expression in cerebellar nuclei after birth

The descent of cerebellar nuclei neurons marks the last migratory step in the formation of cerebellar nuclei. This migratory phase proceeds in a protracted developmental timing from late embryogenesis through early postnatal stages. By P7, three distinct cerebellar nuclei can be clearly distinguished by Meis1 and Meis2 expression along the mediolateral axis. A series of sagittal sections starting at lateral regions through the cerebellar midline, showed many Meis2^{+} cells clustered into three distinct nuclei (Fig. 3-4). Matching of

Figure 3-4 Expression of Meis2 in cerebellar nuclei after birth

A series of sagittal sections from lateral to medial cerebellum show Meis2 expression in neurons of the cerebellar nuclei at P6. (A-E) Meis2 is expressed in the lateral nucleus, the intermediate nucleus, and the medial nucleus. The expression of Meis1 is similar to the one exhibited by Meis2 in the cerebellar nuclei. However, Meis1 is not restricted to CN only as it is expressed by granule cells. Irx3 is also expressed in the cerebellar nuclei at postnatal stages.



Lateral to medial sequence

histological sections and the gene expression data revealed that Meis1 and Meis2 labeled the three subdivisions, namely lateral (dentate) nucleus, the intermediate (interpositus) nucleus, and the medial (fastigial) nucleus. Irx3, Meis1and Meis2 expression persisted in the three cerebellar nuclei through postnatal stages. In turn, Lhx9 expression perdures in the nuclei but Lhx2 is expressed in the EGL. Thus the results show that, after birth, Irx3, Meis1, Meis2 and Lhx9 transcription factors label cerebellar nuclei neurons of the three subdivisions of the cerebellar nuclei.

Discussion

By evaluating multiple criteria, including time of birth, timing of neuronal differentiation, axonal projections, and cell positions over time, we demonstrated that Irx3, Meis1, Meis2, and Lhx2/9 define a cerebellar nuclei neurons during embryogenesis and postnatal stages. Our results present compelling evidence that cell progenitors that give rise to the cerebellar nuclei derive from the ventricular zone. This conclusion is supported by Irx3, Meis1 and Meis2 expression profiles. First, we show that Irx3 is differentially expressed by precursors of the cerebellar nuclei in the VZ and postmitotic cells distributed in the surface, the territory where cerebellar nuclei neurons begin to differentiate. Second, Irx3 expression persists in differentiating

cerebellar nuclei neurons from early embryonic neurogenesis throughout adulthood. Moreover, Irx3 labels projections from the cerebellar nuclei, providing further evidence that Irx3 marks cerebellar nuclei neurons. Third, Meis2, a transcription factor that is also differentially expressed by postmitotic cerebellar nuclei neurons throughout cerebellar ontogeny, marks cell precursors migrating from the VZ to the surface through the wall of the cerebellar anlage (arrows in Fig. 3-1B show Meis2$^+$ cell precursors migrating from the VZ along radial glial fibers). Thus, the differential expression of Irx3, Meis1 and Meis2 in neural precursors migrating along radial glial fibers strongly suggests that progenitors derived from the VZ contribute to the CN.

Recent fate mapping studies have identified that Math1$^+$ progenitors positioned in the aRL contribute to the formation of the cerebellar nuclei. Previous work by Wingate and Hatten (1999) first demonstrated that a subpopulation of precursors within the *Math1*$^+$ cells that give rise to cerebellar granule cells migrate ventrally to establish the lateral basilar pons. The finding that *Math1*$^+$ rhombic lip progenitors migrate outside of the cerebellum has been extended in two recent fate-mapping studies of the cerebellum showing that *Math1*$^+$ cells contribute to a number of brainstem nuclei within the “cerebellar system” (Machold and Fishell, 2005) and to the cochlear nucleus (Wang et al., 2005). In addition, both of these studies show that *Math1+* cells generated in the

aRL after E11.5 migrate into the emerging cerebellar nuclei (Machold and Fishell, 2005; Wang et al., 2005).

Our molecular profiling also revealed that several transcription factors are common to granule cell progenitors and cerebellar nuclei neurons. As it will be discussed later, we found that the transcription factor Meis1 is expressed in cerebellar nuclei neurons and granule cells from early embryonic neurogenesis through postnatal stages. Similarly, Pde1c, Olig1 and Olig2 are expressed in both neural cell types.

The gene expression dynamics show a detailed picture of nucleogenesis within the cerebellum. The expression of TALE (Irx, Meis) transcription factors follow the three steps migratory pathway undertaken by cerebellar nuclei neurons during the formation of the nuclei. First, cerebellar nuclei neurons undergo radial migration to transit from the VZ to the surface, underneath the pia matter. Cerebellar nuclei neurons then migrate tangentially in parallel to the pia and aggregate in the anterior cerebellum. Finally, cerebellar nuclei neurons migrate inward as they segregate into three distinct nuclei that eventually settle in the depth of the cerebellum.

Chapter 4

A map of transcription factor expression in precursors of the cerebellar cortex

Introduction

In the embryonic cerebellum, precursors of the cerebellar granule neurons arise in the aRL and migrate onto the surface of the anlage where they form the EGL. Previous studies established that granule cell progenitors express the bHLH gene atonal 1 (Math1), at E12.5, and that Math1 expression persists in proliferating granule cells postnatally (Ben-Arie et al., 1997). Also, it was shown that transcription factors Zic1, Zic3 and Pax6 mark granule cell progenitors in the EGL (Ben-Arie et al., 1997; Engelkamp et al., 1999; Nagai et al., 1997). In this section, we report that Meis1 is a new marker for granule cell progenitors from embryonic neurogenesis throughout postnatal stages.

Purkinje cells are generated at early stages of embryonic neurogenesis and are a key component of the cerebellar cortex. While a number of studies has shed light on the differentiation, connectivity

and physiology of Purkinje cells at late embryonic and postnatal stages, less is known about the development of this neural cell type when they just leave the germinal neuroephitelium and become postmitotic. Indeed, murine Purkinje cells begin to exit the cell cycle at about E11 (Goldowitz et al., 1997; Miale and Sidman, 1961), but they do not express the classical postmitotic Purkinje cell marker Calbindin until E14.5 (Wassef et al., 1985). To fill this lag in knowledge we aimed to identify transcription factors to be differentially expressed by precursors of the Purkinje cells.

Results

4.1 Meis1 marks granule cell progenitors in the EGL

To follow the emergence of granule cell progenitors from the aRL, we carried out *in situ* localization of *Math1* mRNA. At E12.5, *Math1* expression was restricted to the aRL, which can be visualized in the caudal region of the cerebellar anlage in sagittal sections (Fig. 4-1 A). By E14.5, *Math1* expression persisted in the aRL and was also prominent in the EGL (Fig. 4-1 C).

Our spatiotemporal mapping of transcription factor expression revealed that, in addition to labeling cerebellar nuclei neurons, Meis1 marked cells in the aRL at E12.5 (Fig. 4-1 B). At later stages, when the

cerebellar nuclei neurons had migrated off the surface, Meis1 expression was seen in the EGL, suggesting that Meis1 is expressed by granule cell progenitors (Fig. 4-1 D). To confirm this, we immunostained a *Math1* BAC transgenic mouse line with antibodies against EGFP and Meis1. At E16.5, when formation of the EGL is complete, the vast majority of Math1$^+$ cells in the EGL co-expressed Meis1 (Fig. 4-1 E-G). These findings extend previous studies showing that Math1, Zic1, Zic3 and Pax6 mark granule cell progenitors in the EGL (Ben-Arie et al., 1997; Engelkamp et al., 1999; Nagai et al., 1997), and that Pax2 marks subsets of GABAergic cortical interneurons at early cerebellar neurogenesis (Maricich and Herrup, 1999).

4.2 Meis1 marks granule cells after birth

Our homeodomain protein expression data also illustrate the migratory pathway of granule cells. Briefly, we found that Meis1 is expressed in the aRL at E12.5. From E14.5 onwards, Meis1 expression persisted in the aRL and it appeared in the EGL. After birth, streams of Meis1$^+$ cells located in the EGL migrated inward through the Purkinje cell layer to form the IGL. Meis1 marks granule cells in the IGL both in postnatal and adult tissue, providing further support to the idea that Meis1 is a granule cell marker (Fig. 4-2 C). Interestingly, Meis1 has

Figure 4-1 Meis1 expression identifies granule cell progenitors in the arl and EGL

Sagittal sections of the mouse cerebellum at various embryonic stages. (A) At E12.5, in situ hybridization for *Math1* reveals granule cell progenitors localized in the arL, which is seen occupying the caudal cerebellum. (B) By E12.5, Meis1 is expressed by granule cell progenitors located in the arl (arrow). (C) At E14.5, *Math1* is expressed by granule cell progenitors both in the arl and egl. (D) At E14.5, Meis1 is expressed within the egl. Note: Meis1 is also expressed in the cerebellar nuclei. (E-G) Double immunostaining with EGFP and Meis1 antibodies of an E16.5 *Math1* BAC transgenic mouse reveals that virtually all Math1^{+} granule cell progenitors coexpressed Meis1 in the egl (G= merge of E + F).

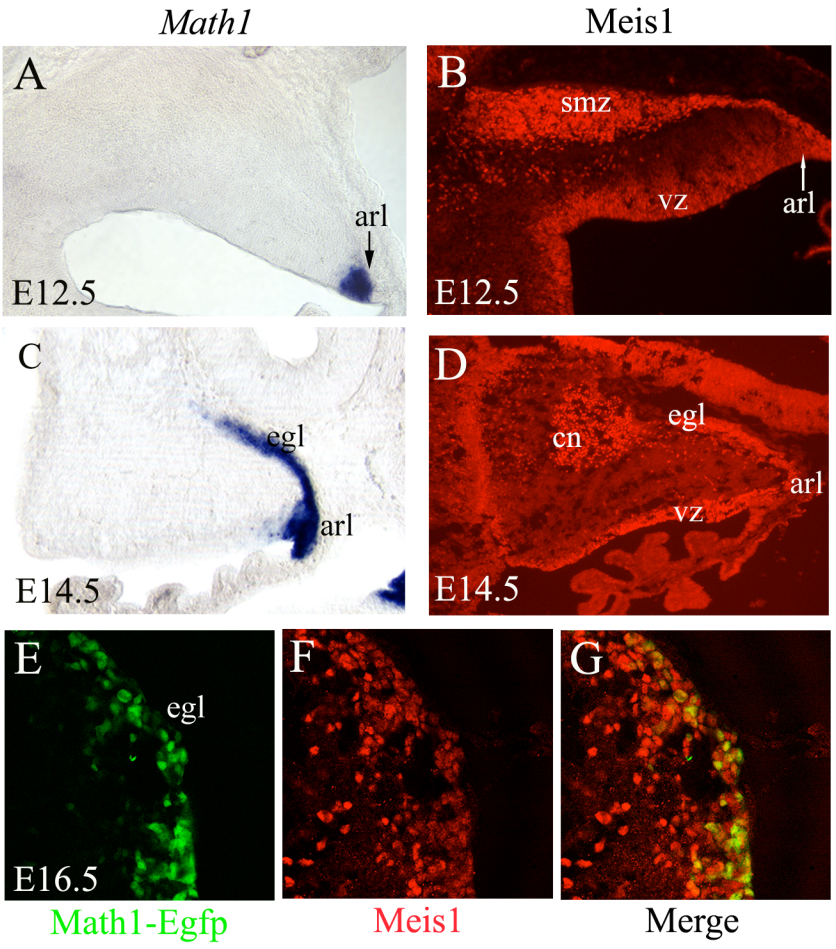
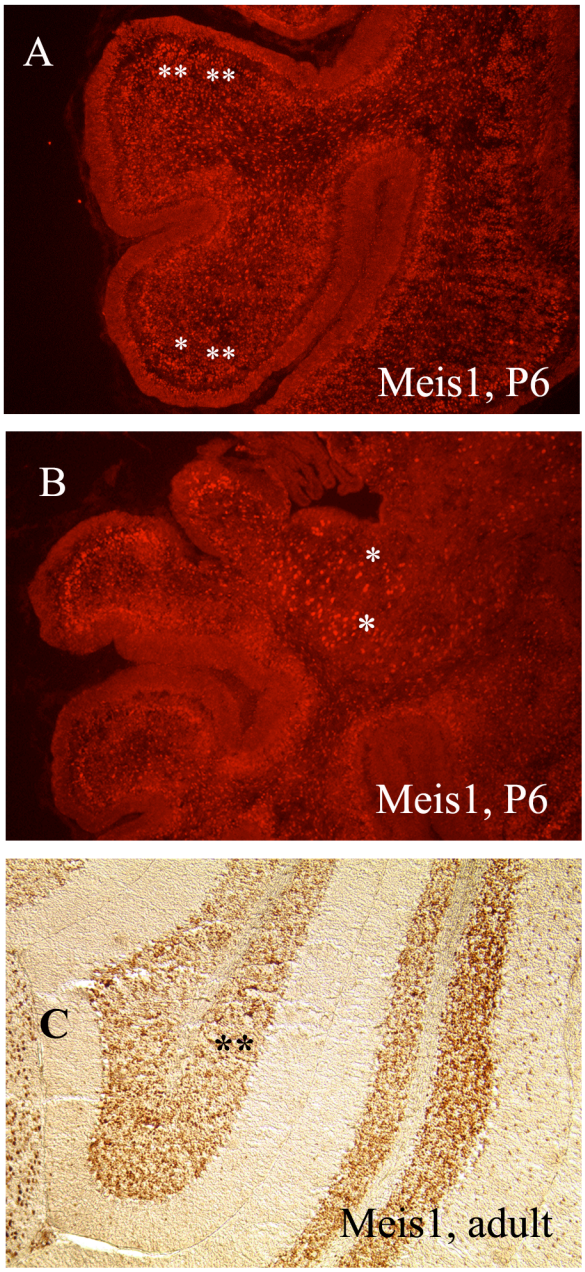

Math1
Meis1
A
arl
E12.5
B
smz
vz
arl
E12.5
C
egl
arl
E14.5
D
cn
egl
arl
vz
E14.5
E
egl
E16.5
F
G
Math1-Egfp
Meis1
Merge

Figure 4-2 Expression of Meis1 after birth

(A) A sagittal section through the P6 cerebellum show that Meis1 is expressed in granule cell progenitors in the EGL and in granule cells migrating to the IGL (asterisks). (B) Sagittal section. Meis1 expression is also detected in the cerebellar nuclei at P6 (asterisks). (C) A sagittal section through the adult cerebellum shows Meis1 expression in mature granule cells located in the IGL. Thus, Meis1 labels granule cell progenitors from early embryonic neurogenesis throughout adulthood.


A
** **
* **
Meis1, P6
B
*
*
Meis1, P6
C
**
Meis1, adult

been found to be highly upregulated in the malignant cerebellar tumors called medulloblastomas, which are generated by a disruption in the regulation of granule cell proliferation (Geerts et al., 2003; Jones et al., 2000).

Discussion

Fate mapping studies by Wingate and Hatten, (1999) first demonstrated that a subpopulation of precursors within the *Math1*$^{+}$ cells that give rise to cerebellar granule cells migrate ventrally to establish the lateral basilar pons. The finding that *Math1*$^{+}$ rhombic lip progenitors migrate outside of the cerebellum has been extended in two recent fate-mapping studies of the cerebellum showing that *Math1*$^{+}$ cells contribute to a number of brainstem nuclei within the cerebellar system (Machold and Fishell, 2005) and to the cochlear nucleus (Wang et al., 2005).

By E12.5, proliferating progenitors arising in the aRL have started to migrate along two pathways, under the influence of distinct attractive and repulsive guidance cues. The first route, taken by the earliest-born progenitors (Gilthorpe et al., 2002; Machold and Fishell, 2005) is a ventral pathway toward the positions where the lateral pontine nucleus (Wingate and Hatten, 1999), and other brainstem nuclei (Gilthorpe et al., 2002; Machold and Fishell, 2005), and the cochlear nucleus will

form (Wang et al., 2005). The second pathway is onto the surface of the cerebellar anlage, where the major pool of progenitors is fated to form the EGL which generates the granule cell of the cerebellar cortex. Studies by Machold and Fishell (2005) and Wang et al (2005) illustrate that some of the *Math1*+ progenitors generated by E10.5 in the aRL migrate onto the surface and follow the earlier pools of CN progenitors, generated in the ventricular zone, into the three emerging cerebellar nuclei (Machold and Fishell, 2005; Wang et al., 2005). Our own molecular profiling shows that Meis1, Pde1c, Olig1 and Olig2 are markers expressed both by the VZ and the aRL. Taken together, these resuts suggest that cerebellar nuclei neurons derive from the two cerebellar germinal sources, the VZ and the aRL.

As reported previously in a number of studies, a second proliferative zone, the aRL, is the site of neurogenesis for several sets of neural progenitors. Progenitors of the other principal neuron of the cerebellar cortex, the granule cell, continue to proliferate until the second postnatal week, as they first form a layer on the surface of the anlagen and then migrate inward to form the internal granule cell layer (IGL). Cells destined to form granule neurons express two classes of transcription factors, namely bHLH (Math1) and TALE (Meis1), as they migrate onto the surface of the anlage, beginning on approximately day E12.

Thereafter, a complex pattern of cell migrations generates the nuclear organization of the cerebellar nuclei, and positions the presumptive cortical neurons above the emerging nuclear structures. *Irx3*$^{+}$*/Meis1/2*$^{+}$*/Lhx2/9*$^{+}$ precursors leave the superficial zone of presumptive CN progenitors beginning on E14. Between E14 and E16, CN progenitors move toward the border of the anlagen before turning and moving down through the developing wall of the anlagen, between the glial fibers that are directing the radial migration of immature Purkinje cells in that period.

4.3 Lhx1 and Lhx5 are expressed by Purkinje cells during embryonic neurogenesis

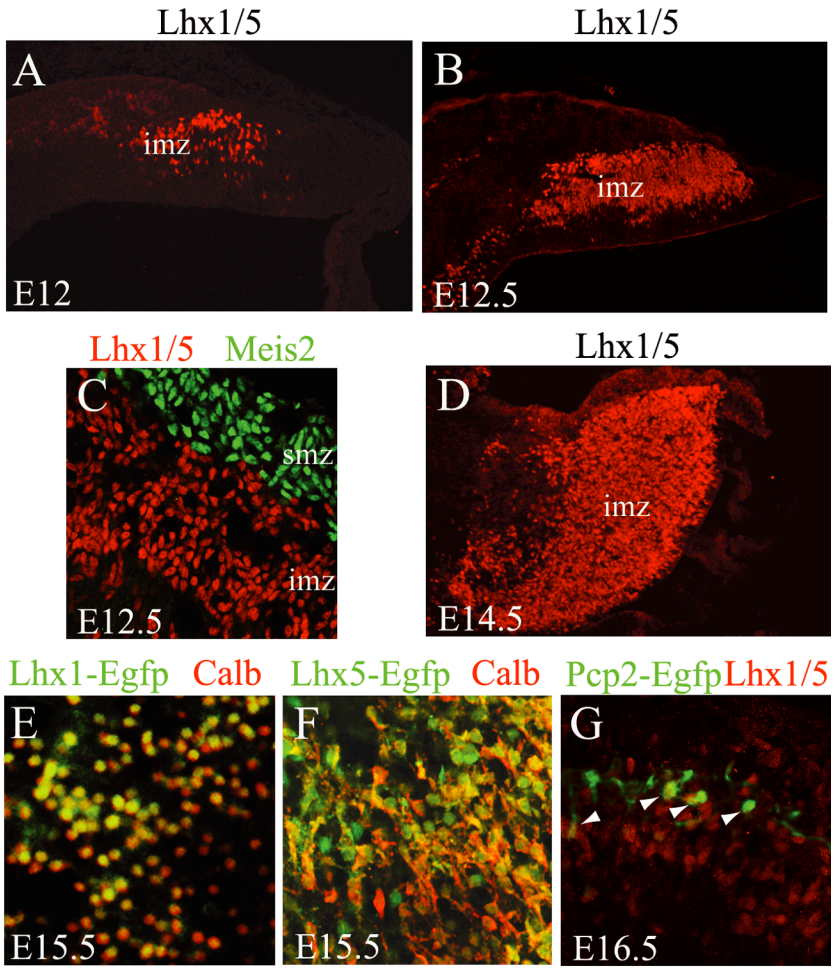
Among the transcription factors we surveyed at E11.5, an antibody specific for Lhx1 and Lhx5 homeodomain proteins (Tsuchida et al., 1994), labeled a few scattered cells located just above the VZ, an intermediate mantle zone, region where the Purkinje cells differentiate (Yuasa et al., 1991). By contrast, Lhx1/5 expression was absent in the VZ at all embryonic stages, consistent with previous studies showing that LIM homeodomain proteins mark postmitotic cells in various neural structures (Fujii et al., 1994; Nakagawa and O'Leary, 2001; Tsuchida et al., 1994). Between E12-E12.5, Lhx1/5 expression became prominent in cells distributed throughout the intermediate mantle zone (Fig. 4-3

A,B). Lhx1/5 staining exhibited a non-overlapping, complementary expression domain to the one defined by Lhx2/9, Irx3, Meis1 and Meis2 in the superficial mantle zone (Fig. 4-3 C; for Lhx2/9 expression see Fig. 3-2 and section 3.1). By E14.5, Lhx1/5 expression persisted within the region that consists of migrating Purkinje cells and cortical interneurons (Fig. 4-3 D). Since Lhx1/5 homeodomain protein expression began at about E11.5 and continued to be restricted to the intermediate zone over time, we hypothesized that early postmitotic Purkinje cells may be defined by the LIM homeodomain proteins Lhx1 and Lhx5.

To confirm the identity of $Lhx1^+$ precursors, we immunostained E15.5 tissue sections from *Lhx1* BAC transgenic mice with the specific Purkinje cell marker, Calbindin (Calb1). Many cells coexpressed Calb1 and Lhx1 in the mantle zone. A small subset of the $Calb1^+$ cells were Lhx1 negative, the reverse was not the case as numerous $Lhx1^+$ cells were Calb1 negative (Fig. 4-3 E). This could reflect the fact that Calbindin labels more differentiated Purkinje cell precursors or that Lhx1 is also expressed by interneurons. At E16.5, Lhx1 expression continued in Purkinje cell precursors and in the EGL. Taken together, these results support the conclusion that Lhx1 is an early marker for Purkinje cells. To examine whether the expression patterns observed with the Lhx1/5 antibody also reflect Lhx5 homeodomain protein

Figure 4-3 The combinatorial expression of Lhx1 and Lhx5 defines Purkinje cell precursors

Sagittal sections through the E12 (A) and E12.5 (B) mouse cerebella show Lhx1/5 expression in prospective Purkinje cells located in the intermediate zone, between the VZ and the surface of the anlage. (C) A transverse section shows that Lhx1/5 expression (red) is restricted to the intermediate zone. Lhx1/5 (red) and Lhx2/9 expression (green) define complementary, non-overlapping territories. (D) A sagittal section of an E14 mouse cerebellum shows that Lhx1/5 expression persisted in the zone where Purkinje cells differentiate. As early as E14 young Purkinje cells are stained with the marker Calbindin, making it possible to correlate Lhx1 expression with this classic marker of differentiation. (E) Two color immunostaining of E15.5 sagittal sections from *Lhx1* BAC mice with antibodies against EGFP and Calbindin (Calb1) shows that Lhx1 and Calb1 colocalized, demonstrating that Lhx1 is a Purkinje cell marker. (F) Similar results were obtained when performing Calbindin immunostaining on *Lhx5* BAC transgenic mice. (G) Two color immunostaining of E16.5 *Pcp2* BAC mice with EGFP and Lhx1/5 antibodies show that Lhx1/5 is expressed by $Pcp2^{+}$ Purkinje cell precursors.


Lhx1/5
A
imz
E12
Lhx1/5
B
imz
E12.5
Lhx1/5 Meis2
C
smz
imz
E12.5
Lhx1/5
D
imz
E14.5
Lhx1-Egfp Calb
E
E15.5
Lhx5-Egfp Calb
F
E15.5
Pcp2-EgfpLhx1/5
G
E16.5

expression in Purkinje cell precursors, we immunostained *Lhx5* BAC transgenic mice with Calbindin. At E15.5, many cells coexpressed Lhx5 and Calb1 in the intermediate mantle zone, confirming that Lhx5 marks Purkinje cell precursors in the developing cerebellum (Fig. 4-3 F). To further investigate the relationship between Lhx1 and Lhx5 transcription factors and Purkinje cells, we immunostained Purkinje cell marker 2 (*Pcp2*) BAC transgenic mice with Lhx1/5 (see Hagen et al., 1996). At E16.5, Lhx1/5 is higly expressed in the intermediate mantle zone. Pcp2 is expressed only by some Purkinje cell precursors differentiating in the mantle zone. Most $Pcp2^{+}$ cells coexpressed Lhx1/5, but the majority of the cells expressing Lhx1/5 were Pcp2 negative (Fig. 4-3 G). Taken together, our findings show that the combinatorial expression of Lhx1 and Lhx5 defines Purkinje cell precursors from the time they leave the VZ.

4.4 Radial migration of Purkinje cell precursors

Although the development of Purkinje cells has previously been inferred using birthdating techniques and molecular markers (Feirabend et al., 1985; Lin and Cepko, 1998; Millen et al., 1995; Yuasa et al., 1991), absence of early markers for Purkinje cell precursors has precluded direct observations of their migratory pathway when they just become postmitotic. Here, we show that $Lhx1/5^{+}$ Purkinje cell

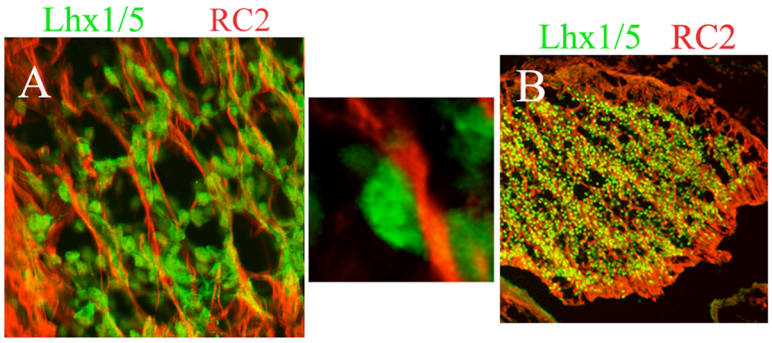


Fig. 4-4 Radial migration of Purkinje cell precursors

(A, inset) Transverse tissue sections show that Lhx1/5 is strongly expressed by neurons migrating closely opposed to $RC2^{+}/BLBP^{+}$ radial glial processes at E12.5. (B) Numerous $Lhx1/5^{+}$ cells were evident migrating along the $RC2^{+}/BLBP^{+}$ radial glial fiber system at E14.5.

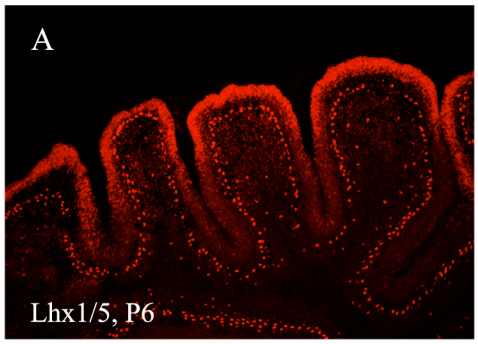
precursors leave the VZ associated to the radial glia. In transverse tissue sections, Lhx1/5 is strongly expressed by migrating neurons closely opposed to $RC2^+/BLBP^+$ radial processes at E12.5 (Fig. 4-4 A, inset). Numerous $Lhx1/5^+$ cells were evident along the $RC2^+/BLBP^+$ radial glial fiber system at E14.5 (Fig. 4-4 B). These results provide direct evidence for the long-standing assumption that Purkinje cell precursors migrate away from the VZ along radial glia.

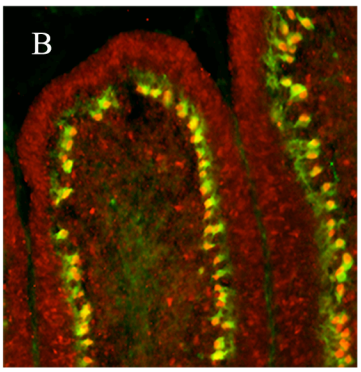
4.5 Lhx1/Lhx5 expression after birth

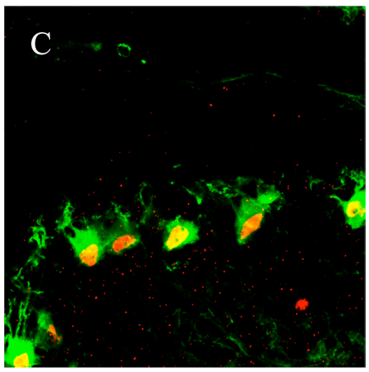
During late embryogenesis through postnatal stages, expression of Lhx1 and Lhx5 is dynamic. At E16.5, Lhx1 is expressed in Purkinje cells and in the EGL and this expression profile persisted through postnatal stages. At P6, Lhx1 expression is detected in Purkinje cells arranged in a monolayer (Fig. 4-5 A). Double immunostaining with Lhx1/5 and Calb1 antibodies, showed that these markers colocalized in Purkine cells at P7 (Fig. 4-5 B,C). Lhx1/5 is also expressed by interneurons distributed in the EGL and IGL postnatally (Fig. 4-5 B). Notably, Lhx1/5 continued to be expressed in Purkinje cells in adults (Fig. 4-5 D).

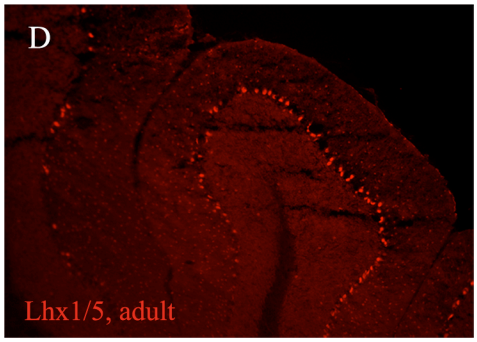
Figure 4-5 Expression of Lhx1/5 after birth

(A-D) Sagittal sections of postnatal and adult cerebella. (A) At P6, Lhx1/5 expression marks Purkinje cells arranged in a monolayer. Lhx1/5 is also expressed in interneurons that occupy the EGL and IGL at this stage. (B, C) Two color immunofluorescence shows that Calb1 and Lhx1/5 colocalize in Purkinje cells. (D) In the adult cerebellum, Lhx1/5 is expressed in Purkinje cells and interneurons positioned in the molecular layer and IGL.


A
Lhx1/5, P6


B
Lhx1/5
Calb1


C
Lhx1/5
Calb1


D
Lhx1/5, adult

Discussion

The Purkinje cell, owing to its central role in cerebellar physiology, is one of the best-studied neurons in the vertebrate brain. While extensive gene profiling has revealed as many as several thousand genes in postnatal Purkinje cells, insight on the earliest steps in Purkinje cell development has been lacking. The present study suggests a role for at least two members of the LIM homeodomain family of transcription factors (Lhx1/5) in early steps in PC differentiation between cell cycle exit (E11.5-13) and the expression of the "signature markers" Calb1, Pcp2 (L7) (E14.5-E16). The present study used these early markers for Purkinje cell precursors, combined with specific markers for radial glia, to demonstrate that early postmitotic PC progenitors migrate away from the germinal zone along the glial fiber pathway. Previous studies were restricted to later stages (E15), after the onset of Calbindin expression. Moreover, prior studies used vimentin, a general marker for neuronal and glial fibers, rather than RC2 or BLBP to label radial glia. The results on the migratory pathway of PC precursors demonstrate that this key cerebellar neuron undergo glial-guided migration.

Chapter 5

Cerebellar neurons in the chick are defined by the same combination of transcription factors that define them in the mouse

Results

To examine whether the pattern of transcription factor expresion we observed for murine cerebellar neurons was conserved in other vertebrates, we carried out parallel studies in chick embryos. Previous work showed that the histology and morphogenesis of the cerebellum is highly conserved between avian and mammalian species (Ramon y Cajal, 1911). We found that the same markers that identified murine cerebellar nuclei neurons, Purkinje cells and granule cells labeled the corresponding chick progenitor cell populations. In the e4.5 (HH24) chick cerebellum, staining of transverse sections shows that Lhx2/9 expression was expressed by postmitotic cells located in the surface of the anlage (Fig. 5-1 A). By contrast, Lhx1/5 defined postmitotic cells located above the VZ (Fig. 5-1 B). At e5 (HH26), increased numbers of postmitotic cells are seen in the anlage. Sagittal sections show that Lhx2/9 expression persisted in prospective cerebellar nuclei neurons

located in the surface zone (Fig. 5-1 C), whereas that Lhx1/5 expression was prominent in the underlying zone of prospective Purkinje cells zone (Fig. 5-1 D). Thus, as seen in the mouse cerebellum, the expression of LIM homeodomain proteins Lhx2/9 and Lhx1/5 defines complementary, non-overlapping postmitotic territories within the cerebellar anlage zone (Fig. 5-1 E). At e7 (HH31), Lhx2/9 and Lhx1/5 expression continued to be restricted to prospective cerebellar nuclei neurons and Purkinje cell precursors, respectively (data not shown).

To examine the expression patterns of *Irx3* and *Meis1* in the chick cerebellar anlage, we carried out whole mount in situ hybridization analysis with specific RNA probes, at stages e5 and e7. In the e5 chick cerebellum, in situ hybridization to *Irx3* and *Meis1* mRNAs revealed cells distributed throughout the surface of the anlage (data not shown). The expression of Irx3 reveals that cerebellar nuclei neurons initially migrate radially from the VZ to the surface of the anlage (Fig 5-2). At e7, flat-mounted tissue shows that *Irx3* and *Meis1* are expressed by cerebellar nuclei neurons which have begun to cluster in the depth of the cerebellar anlage zone (Fig. 5-1 F,G, asterisks). Despite the cerebellar nuclei precursors do not cluster until e7, cerebellar nuclei neurons begin to project axonal efferences at about e4 (Fig. 5-3).

Figure 5-1 Expression of LIM and TALE transcription factors defines nuclear neurons, Purkinje cells and granule cell progenitors in the chick.

(A) Transverse sections show that Lhx2/Lhx9 is expressed in cerebellar nuclei precursors (CNp) in the surface of the e4.5 chick cerebellum (arrow). (B) By contrast, Lhx1/5 expression demarcates the zone where PCp differentiate (arrow). Sagittal sections of e5 chick cerebellum show that Lhx2/Lhx9 expression persists in CNp (C), whereas that Lhx1/5 is expressed by Purkinje cell precursors (D). (E,F) Flat-mount preparations of e7 cerebellum hybridized with probes to Irx3 and Meis1. (E) *Irx3* expression is restricted to prospective nuclear neurons coalescing in the ventral region of the cerebellum (medial region in the flat-mount). (F) Likewise, *Meis1* expression shows prospective nuclear neurons during nuclei formation. Note that *Meis1* also defines granule cells progenitors in the arl (the lateral edge, see arrow).

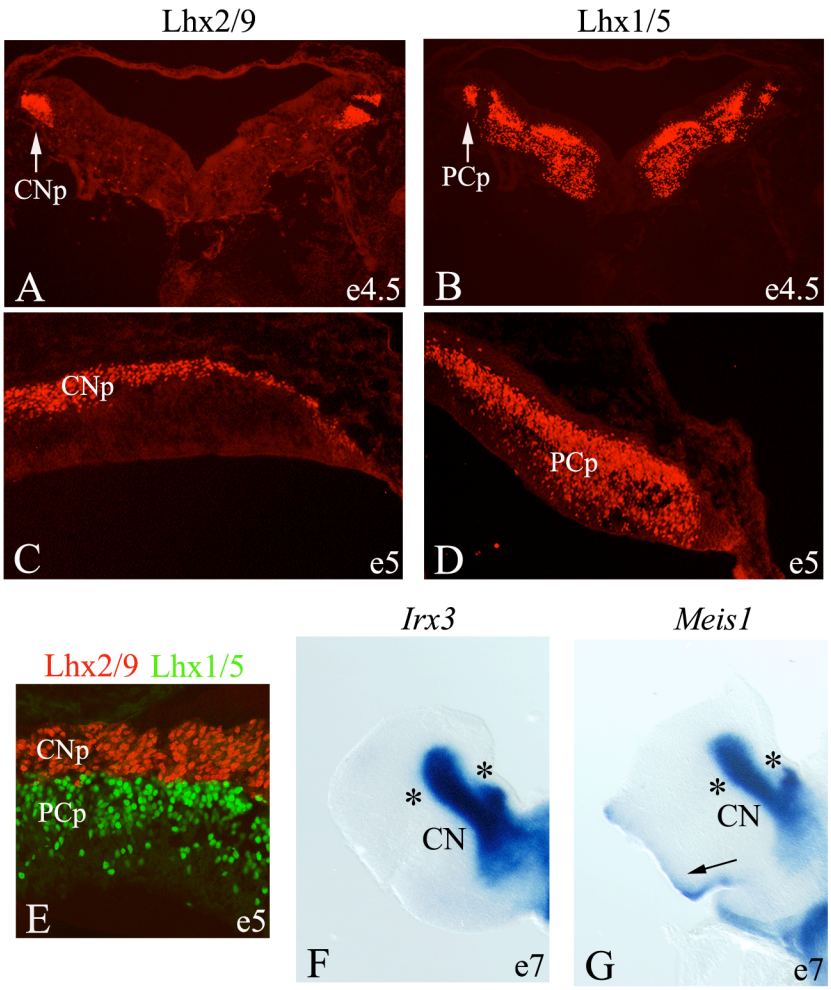

Lhx2/9
Lhx1/5
CNp
PCp
A
e4.5
B
e4.5
CNp
PCp
C
e5
D
e5
Lhx2/9 Lhx1/5
CNp
PCp
E
e5
Irx3
Meis1
CN
CN
F
e7
G
e7

Figure 5-2 Migration of the cerebellar nuclei precursors from the VZ to the surface in the chick

The cerebellum of chicken embryos was targeted by *in ovo* electroporation with an expression vector driving Irx3 expression and other driving EGFP reporter expression. A coronal section immunostained with Irx3 antibody shows columns of $Irx3^{+}$ (red) /$EGFP^{+}$ (green) migrating cells transiting from the VZ to the surface of the cerebellar anlage. Pial-directed migration of cerebellar nuclei precursors is observed.

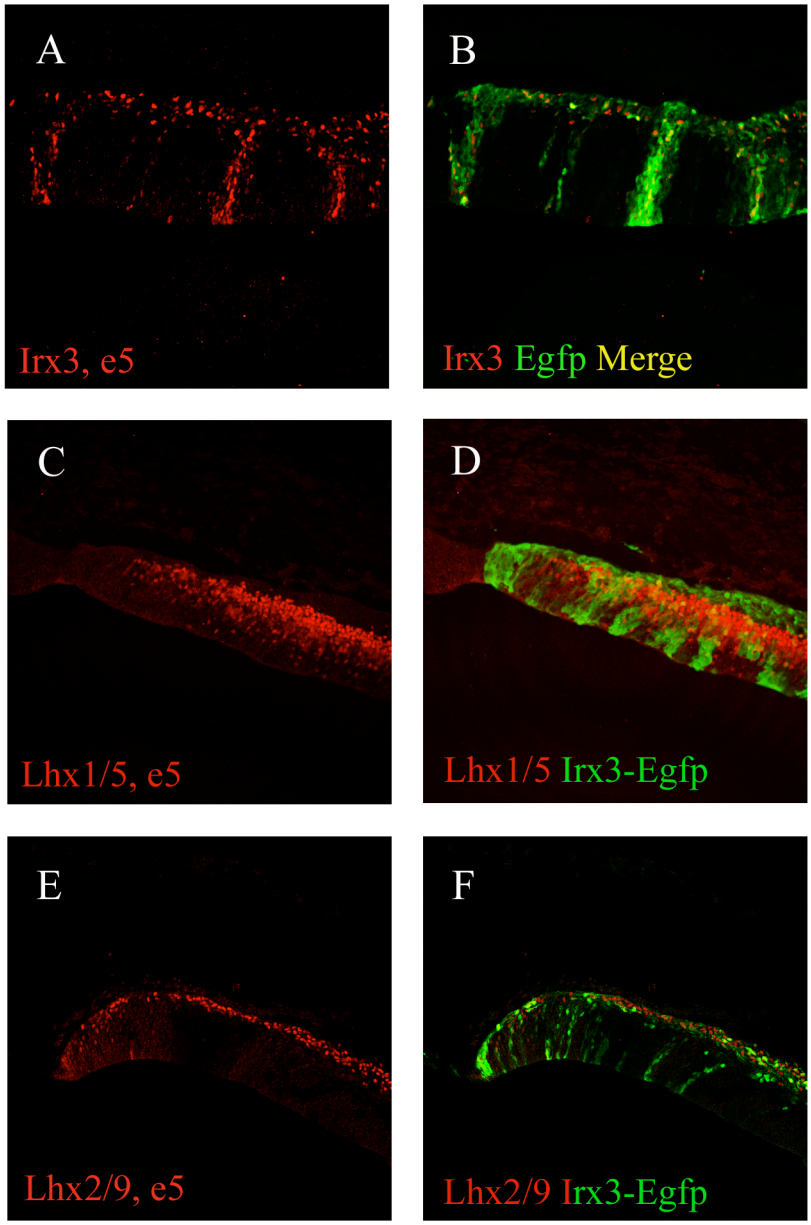
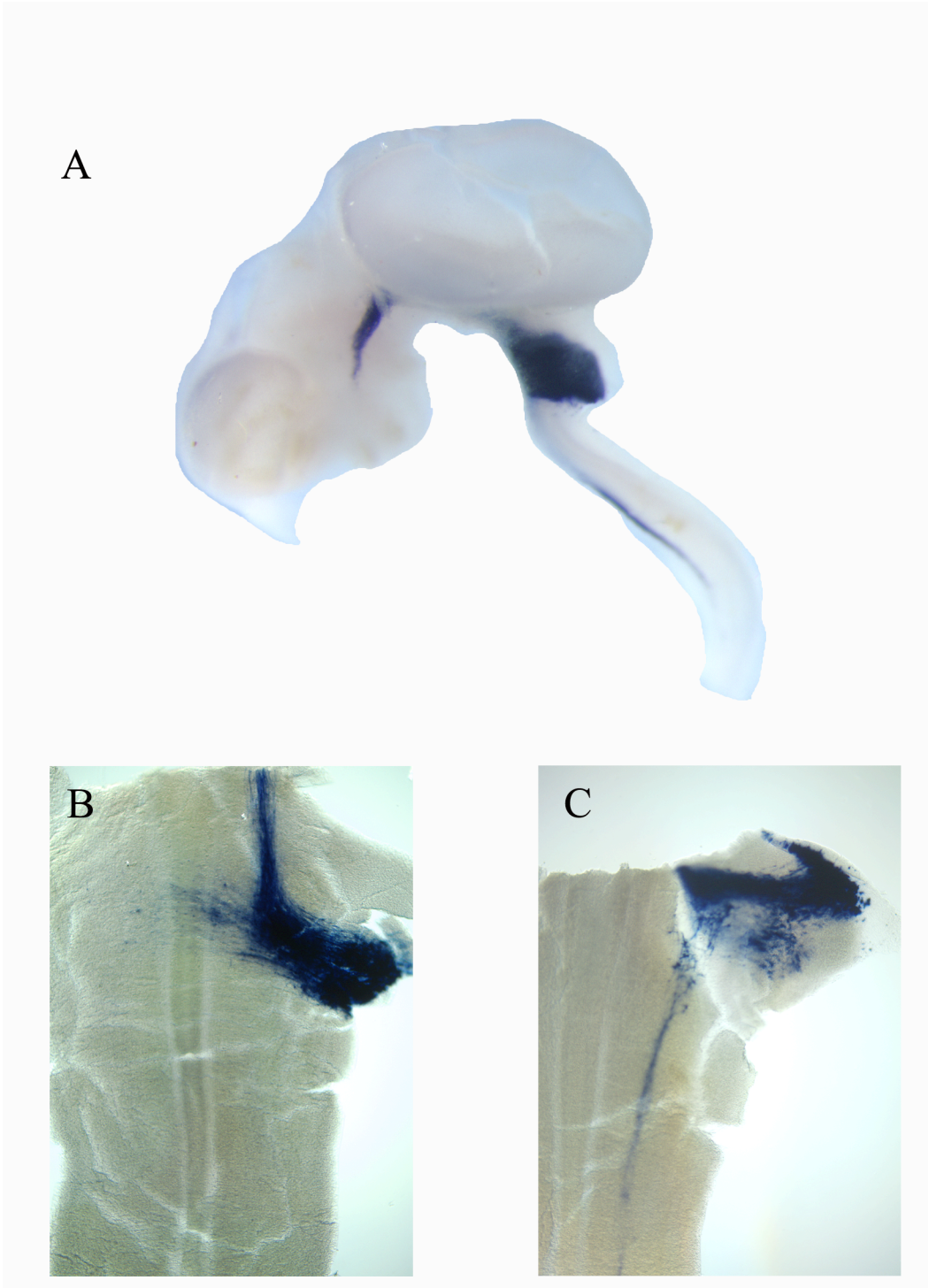

A
Irx3, e5
B
Irx3 Egfp Merge
C
Lhx1/5, e5
D
Lhx1/5 Irx3-Egfp
E
Lhx2/9, e5
F
Lhx2/9 Irx3-Egfp

Figure 5-3 Precursors of the cerebellar nuclei project efferences at early embryonic stages

The cerebellum of chicken embryos was *in ovo* electroporated with an alkaline phosphatase reporter expression vector driven by Elongation factor 1 promoter to strongly label cerebellar axonal projections. (A) A whole mount e5 chicken brain shows cerebellar projections to the thalamus and spinal cord. These projections can be detected as early as e4. A flat-mount preparation of e5 in ovo electroporated tissue with PLAP-EF1 shows in greater detail axonal projections from the cerebellum to the thalamus (B) and spinal cord (C).


A
B
C

Two incipient cerebellar nuclei could be distinguished in our flat-mount preparations at this stage.

Although several nuclear nuclei have been reported in adult birds (Ramon y Cajal, 1911), only two nuclei are evident in the e10 chick cerebellum (Marin and Puelles, 1995). Taken together, these results show that histogenesis of the cerebellar nuclei have similarities in avian and mammalian embryos.

Our in situ detection of *Meis1* mRNA also revealed granule cell progenitors in the aRL at e7 zone (Fig. 5-1 G, arrow). Thus, Meis1 expression defines both cerebellar nuclei neurons and granule cell progenitors. We conclude that an evolutionary conserved transcription factor profile marks cerebellar nuclei neurons, Purkinje cells and granule cells in the chick and mouse cerebellar anlagen.

Discussion

The results described above show that cerebellar nuclei neurons segregate into distinct nuclei, whereas Purkinje cells and granule cells form distinct laminae during cerebellar histogenesis. These complex morphogenetic events, namely nucleogenesis and corticogenesis, involve the coordinated movement of distinct neural cell populations. Our atlas of transcription factor expression provides a spatiotemporal

map of the migratory pathway of the precursors of the cerebellar nuclei and overlying cerebellar cortex during the ontogeny of the chick and mouse. The mechanisms that control this spatiotemporal expression of transcription factors and concomitant programs of gene expression that characterize the cerebellar cell classes can now be analyzed by detailed lineage tracing and studies of transcription factor function(s) in cerebellar cells.

Chapter 6

Discussion and future prospects

Onset of transcription factor expression and gene expression dynamics

This study had contributed to a molecular understanding of cerebellar neurogenesis (Fig. 6-1 A,B). We provide evidence that supports the idea that the onset of transcription factor expression follows a precise temporal sequence that coincides with the temporal course in which cerebellar nuclei neurons, Purkinje cells and granule cell progenitors emerge from the cerebellar germinal neuroepithelium. Nuclear neuron markers Irx3 and Meis1 are already expressed in the VZ by E10; then, Meis2 expression begins in cell precursors migrating to the superficial mantle zone, before E10.25, while the expression of LIM homeodomain proteins Lhx2/9 begins at E10.25 (Fig. 6-2). By contrast, the onset of expression of the LIM homeodomain proteins Lhx1/5 by Purkinje cell precursors is about E11.5 (Fig. 6-2). Thus, a specific program of TALE and LIM homedomain proteins appear to mark the sequential emergence of the three principal neural classes of the cerebellum.

Figure 6-1 Schematic summary of transcription factor expression in the cerebellum at E12.5 and E14.5

(A) At E12.5, the expression of Irx3 and Meis1 mark prospective nuclear neuron progenitors in the VZ. As they reach the surface of the anlage they continue to express Irx3 and Meis1 but also Meis2 and Lhx2/9. Meis1 is also expressed by granule cells in the aRL. In turn, Lhx1 and Lhx5 are expressed by Purkinje cell precursors, just above the VZ. (B) At E14.5, these transcription factors show the cell migration dynamics of precursors of the cerebellar nuclei, Purkinje cells and granule cells.

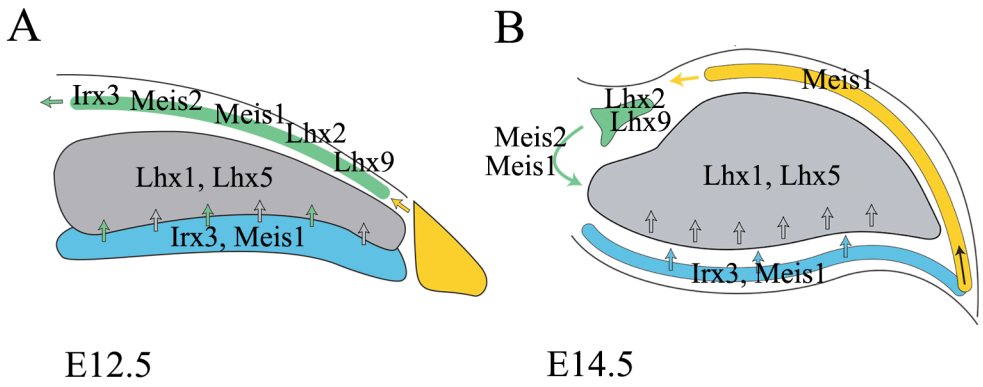
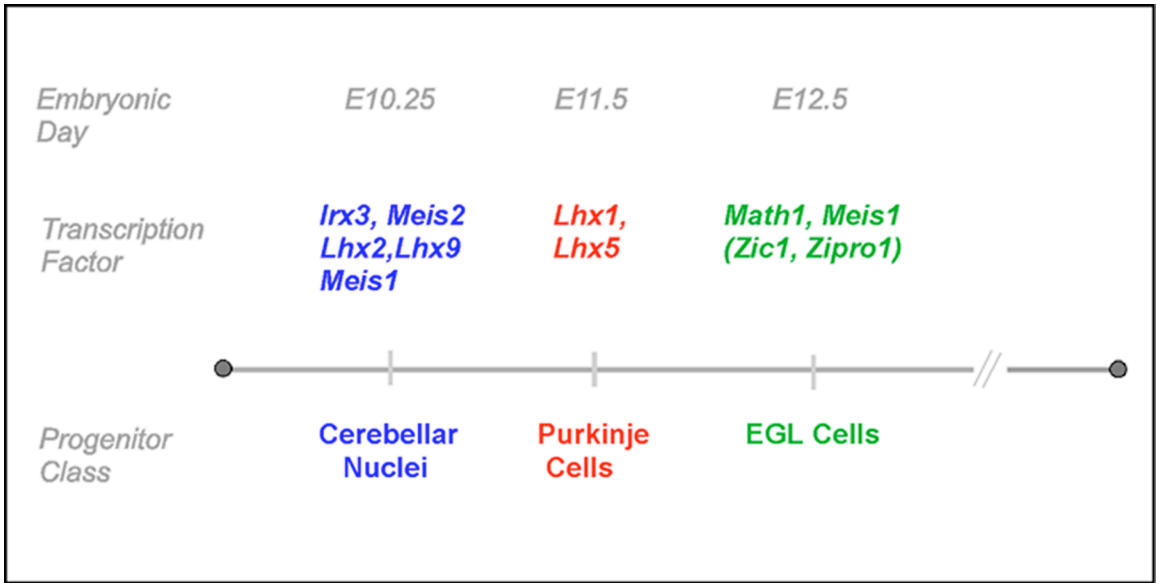

A
Irx3
Meis2
Meis1
Lhx2
Lhx9
Lhx1, Lhx5
Irx3, Meis1
B
Meis1
Lhx2
Lhx9
Meis2
Meis1
Lhx1, Lhx5
Irx3, Meis1
E12.5
E14.5
Cerebellar nuclei neurons
Purkinje cells
Granule cell progenitors
Cell Progenitors in the Ventricular Zone

Figure 6-2 Time-Line of Transcription Factor Expression in Precursor Cell Populations of the Embryonic Cerebellar Anlage

A time line from the day of closure of the neural tube (E9.5), at left, to birth, at right, is given, with the period of cerebellar histogenesis expanded. The time of expression of transcription factors for each class of neural progenitor is given above the line. Progenitors of the neurons of the cerebellar nuclei (blue) are the first pool of progenitors, generated on E10.25, followed by the progenitors for the cortical Purkinje cells (red), on E11.5, and the movement of progenitors of the cortical granule cells (green) as well as progenitors destined for the cerebellar nuclei, pre-cerebellar nuclei and other brainstem nuclei of the “cerebellar system” onto the surface of the anlage, beginning on about E12.5. Between E12.5 and E16, EGL cells spread over the surface of the anlagen. Between E13 and E15, CN precursors move off of the surface and form the cerebellar nuclei.


Embryonic Day
E10.25
E11.5
E12.5
Transcription Factor
Irx3, Meis2
Lhx2,Lhx9
Meis1
Lhx1,
Lhx5
Math1, Meis1
(Zic1, Zipro1)
Progenitor Class
Cerebellar Nuclei
Purkinje Cells
EGL Cells

Homeodomain protein expression patterns and cerebellar histogenesis

Although previous studies showed that the isthmic gene regulatory network that governs the patterning of the cerebellar territory is evolutionary conserved in vertebrates, little was known about how individual neural cell precursors are allocated to distinct nuclear and cortical fates in the developing cerebellum. This work addressed this issue and led to four important conclusions: first, a principle that emerge is that the combinatorial expression of transcription factors marks distinct neural subtypes in the cerebellar anlage. Second, our results on cerebellar histogenesis are in general agreement with previous neuroanatomical and birthdating studies that described similar histology and neurogenetic timetable in the chick and mouse cerebellar anlagen. Third, our detailed account of the time of origin and migratory pathways of cerebellar neurons shows that similar molecular programs control the emergence of the three principal neural classes in the avian and murine cerebella. Fourth, the concerted cell movements that drive the assembly of neurons into the cerebellar nuclei and overlying cerebellar cortex were similar in both model organisms.

The present study provides molecular evidence, consistent with the neuroanatomical literature, for the general idea that different combinations of transcription factors specify the principal classes of

neurons in different brain structures. Our findings show that the histogenesis of the cerebellum occurs via the stepwise generation of progenitor cells that express, timely, selective members of the TALE, LIM and bHLH transcription factor families. A particularly interesting finding is the multi-step migration of neural cell precursors involved in the formation the cerebellar nuclei and the cerebellar cortex. The definition of a set of molecular markers to visualize cerebellar nuclei neurons and Purkinje cells allowed us to demonstrate that both neural cell types move out of the ventricular zone guided by RC2/BLBP^{+} radial glial fibers in their transit to the differentiating mantle zone.

Neuroanatomical studies described the basic histology of the developing cerebellar anlagen (Feirabend et al., 1985; Yuasa et al., 1991; Goldowitz et al., 1997; Altman and Bayer, 1985a; Bourrat and Sotelo, 1986). Our data show that the histological organization of the embryonic cerebellum is similar in chicken, mouse and rat (see Feirabend et al., 1986; Altman and Bayer, 1985a; this study). In particular, previous work proposed that the cerebellar germinal neuroephitelium was subdivided into the ventricular zone (VZ) and the anterior rhombic lip (aRL). The ventricular zone along the IVth ventricle would be the source of cerebellar nuclei neurons, the Purkinje cells and cerebellar interneurons (Dino et al., 2000; Laine and Axelrad, 1994; Laine and Axelrad, 2002; Nunzi et al., 2001; Palay and Chan-Palay,

1974b), whereas the aRL would give rise to granule cells (Harmark, 1954; Hanaway, 1967; Wingate and Hatten, 1999; Lin and Cepko, 1998). In addition, the differentiating zones of the embryonic cerebellum were subdivided into three, namely a region where cerebellar nuclei neurons would undergo differentiation, a region where the development of Purkinje cells and interneurons would occur, and a region containing granule cells undergoing migration.

Although classical ^{3}H–birthdating studies have suggested parallel plans of cerebellar histogenesis in mammals and birds, they did not reveal fine details concerning the precise location and timing of the generation of the distinct cerebellar cell classes. The present study provides the first evidence that similar molecular programs control the emergence of the three principal neural classes at the time when neurogenesis begins in the avian and murine cerebella. Our detailed correlative analysis of the temporal expression of transcription factors and the timing of generation of the distinct cerebellar cell classes provides key new insights into how cerebellar histogenesis is coordinated at early stages of brain development. Our own molecular profiling support the timetable of generation that was previously described for cerebellar nuclei neurons, Purkinje cells and granule cells in the mouse, but also contributed to define the precise timing of various cellular events, including cell proliferation, cell migration and settling in

the mantle zone. For example, precursors of the cerebellar nuclei begin to leave the germinal neuroepithelium at E10-E10.25, and form a transient layer in the surface of anlage until stage E12.5, when a wave of granule cell progenitors emerges from the aRL and sweeps up over the surface to form the EGL. After that, cerebellar nuclei neurons aggregate into distinct clusters or nuclei. Purkinje cells, in turn, begin to migrate out the ventricular zone at E11, to assume a laminar arrangement which is further refined during development. Collectively, we defined the time at which the major cerebellar cell classes leave the germinal neuroepithelium and become postmitotic as well as the spatio-temporal dynamics of cell population movements.

Previous work proposed that the ventricular zone (VZ) along the IVth ventricle generates neurons of the cerebellar nuclei, the Purkinje cell and cerebellar interneurons (Dino et al., 2000; Laine and Axelrad, 1994; Laine and Axelrad, 2002; Nunzi et al., 2001; Palay and Chan-Palay, 1974b; Feirabend et al., 1985; Lin and Cepko, 1998; Millen et al., 1995; Yuasa et al., 1991), nevertheless, the absence of early markers for precursors of Purkinje cells and the cerebellar nuclei has precluded direct observations of their migratory pathway when they just become postmitotic. Here, we show that Lhx1/5^{+} Purkinje cell precursors leave the VZ closely opposed to RC2^{+}/BLBP^{+} radial processes as they migrate to the mantle zone as early as E11.5. These results provide

direct evidence for the long-standing assumption that Purkinje cell precursors migrate away from the VZ along radial glia. Likewise, our results present compelling evidence that cell progenitors that give rise to the cerebellar nuclei derive from the VZ. As Irx3 was expressed by proliferative cells in the VZ and postmitotic cells located in the surface, these experiments allowed, for the first time, direct visualization of columns of prospective cerebellar nuclei neurons moving from the VZ to the surface along the $RC2^{+}$/$BLBP^{+}$ radial glial fibers. Taken together, these findings on the migratory pathway of CNP and PCP, together with both classical and real time imaging of granule cell migration, demonstrate that all three principal classes of cerebellar neurons undergo glial-guided migration (Wingate and Hatten, 1999; Koster and Fraser 2001).

It has been shown that neurons of hindbrain nuclei of the "cerebellar system" are also generated from progenitors located in the aRL, the source of cerebellar cortical granule neurons (Hallonet 1990; Alder et al., 1996; Wingate and Hatten, 1999; Landsberg et al., 2005). Chick-quail fate mapping studies of "the cerebellar system", which includes the cerebellum and the pre-cerebellar brainstem nuclei, first revealed that the progenitor cell population, which emerges from the rhombic lip, is far more complex than previously recognized (Wingate and Hatten, 1999). Over the past several months, mouse genetic fate-

mapping studies have extended those earlier findings to show that progenitors derived from the aRL generate precursor populations for both the cerebellar nuclei and the EGL of the cerebellar cortex, as well as cells of the vestibular nucleus and pre-cerebellar nuclei (Fink et al., 2006; Landsberg et al., 2005; Machold and Fishell, 2005; Wang et al., 2005; Wingate, 2005). It is now evident that the EGL includes cells that have multiple fates. Importantly, subpopulations of EGL cells, generated as early as E10.5 in the aRL (Machold and Fishell, 2005) migrate from the surface of the anlage to contribute to the formation of the cerebellar nuclei, and to brainstem nuclei that project axons to the cerebellum. Machold and Fishell, 2005 went to show on their fate mapping analyses that Math-1-expressing cell progenitors give rise to distinct neuronal populations over time. For example, prior to E12.5 early generated Math1$^+$ cells give rise to some cerebellar nuclei neurons as well as neurons of various nuclei in the hindbrain, including components of the mesopontine cholinergic system in the adult. Between E12.5-E17, Math1$^+$ cells give rise to granule cells, process regulated by inductive signals coming from the roof plate. The new markers that we have identified will aid in the further molecular dissection of these progenitor subpopulations and their site of origin.

Transcription factor expression patterns define cerebellar nuclei neurons, Purkinje cells and granule cells

Our study demonstrated that precursors of cerebellar nuclei neurons and immature Purkinje cells migrate away from the VZ along the radial glial fiber system. Similar to cell progenitors in higher cortical areas, precursors of the cerebellar nuclei neurons (Irx3$^+$, Meis1$^+$, Meis2$^+$) and Purkinje cells (Lhx1$^+$, Lhx5$^+$) appear to migrate along a system of RC2/BLBP$^+$ radial glial fibers during their transit from the ventricular zone lining the IVth ventricle to the differentiating mantle zone. As neuronal differentiation proceeds in the cerebellum, Lhx1/5$^+$ Purkinje cell precursors in the intermediate mantle zone express the classical Purkinje cell markers Calbindin and Pcp2. Cerebellar nuclei neurons remain in the surface of the anlage until a wave of granule cell progenitors emerges from the aRL and sweeps up over the surface at about E12.5. This spreading of granule cells progenitors (marked by Math1 and the new marker Meis1) away the aRL to form the EGL seems to occur in concert with the rostrally directed tangential migration of cerebellar nuclei neurons. Subsequently, cerebellar nuclei neurons cluster into distinct nuclei which migrate inwardly to settle underneath the cerebellar cortex at late embryonic stages. Thus, unique combinations of transcription factors define distinct cerebellar neurons

and demarcate the boundaries of the territories where these distinct neural cell classes differentiate.

Future Prospects

The cerebellum consists of a discrete number of distinct cell types that are well defined morphologically. The generation of these cerebellar cell classes proceeds in an orderly manner within the germinal neuroepithelium during embryogenesis. These features make the developing cerebellum an attractive system to study cell-specific expression of genes. Our map of transcription factor expression that identify precursors of cerebellar nuclei neurons, Purkinje cells and granule cells from embryonic neurogenesis throughout postnatal stages will provide the starting point for functional analysis of the role of various transcription factors in neural cell proliferation and differentiation as well as neuronal migration.

Knock-out mouse studies using null alleles of selected genes would be critical to elucidate functions of individual genes. Regarding the development of Purkinje cells, the generation of knockout mice targeting the transcription factors Lhx1 and Lhx5 would be useful to see which aspects of Purkinje cell differentiation are affected by these LIM genes. However, mice harboring a null allele could prove not to be very

informative as the *Lhx1* and *Lhx5* may act in a combinatorial manner to define aspects of Purkinje cell development. In that sense, engineering *Lhx1* and *Lhx5* compound knockout mice can provide a mutant phenotype for more comprehensive analysis.

Genetic fate mapping strategies based on site-specific recombinases in mice has been a powerful tool to track the integration of molecularly defined cell progenitors generated early during embryogenesis into adult neural structures. The use of fate mapping with Cre- or Flp-mediated recombination has used gene reporters that permanent mark selective cell progenitor populations for delineating the adult structures that arise from a gene expression domain earlier during embryogenesis (Dymecki and Tomasiewicz, 1998). This approach was used to determine the adult derivatives of neural progenitors which transiently express *Wnt-1* during the development of the posterior rhombic lip (pRL) (Dymecki and Tomasiewicz, 1998). High-level *Wnt1* expression merked the progenitor cells within the pRL fated to send mossy fiber axons to granule cells within the cerebellum (Rodriguez and Dymecki, 2000). An interesting alternative is the use of mice engineered for ligand-inducible Cre-mediated recombination. This allow to mark groups of cells at specific embryonic time points in transgenic mice (6). Recently, an intersectional genetic fate-mapping was used to study the development of the cochlear nuclear complex. This approach

allowed to selectively mark cell progenitors at the intersection of two molecularly defined regions, making it possible to define the fates that cell progenitors generated in the pRL and adjancent territories contribute to the cochlear nuclear complex (Farago et al., 2006).

The use of mouse genetics will also contribute significant insights into the cell lineage relationships among the distinct cerebellar cell classes. Previous cell lineage studies focused on the cerebellum used a genetic approach based on the LaacZ clonal method of cell labeling in combination with the neuron-specific enolase promoter to ensure that most labeled β-gal+ cells in the postnatal cerebellum would derive from neuronal ancestors generated back around E6.5 (Mathis and Nicolas, 2003). The laacZ method allows long-term labeling of clones of cells, providing key insights on the distribution of neural progenitors from early stages of brain development throughout histogenesis. This study found lineage relationship between Purkinje, Golgi, and molecular layer neurons in clustered small clones restricted to the cerebellar cortex, suggesting that individual progenitors located in the VZ are multipotent and, give rise sequentially to distinct cell types. Interestingly, they also identified a pool of dividing progenitors which seem to be fated exclusively to generate cerebellar nuclei neurons (Mathis and Nicolas, 2003). Using this method the precise time of birth of the clones is unknown, but correlative analyses with fate mapping

techniques can allow to infer the timing and location of the labeled progenitors. Cell lineage analysis using Cre lines will allow lineage analysis studies to determine with precision the mature cell types to which subsets of mitotic progenitor cells or posmitotic precursors give rise. Within the cerebellum, cell lineage and fate mapping techniques can be applied to follow the development of cell progenitors located in the VZ. Our molecular profiling revealed that Irx3-expressing cells are distributed throughout the VZ and the postmitotic region where cerebellar nuclei neurons differentiate. We also have preliminary results showing that Irx2 is expressed both in the ventricular zone and the postmitotic region where Purkinje cell differentiate. In the postmitotic region Irx2 seems to have a complementary expression pattern to the one exhibited by Irx3. It may be that Irx3 and Irx2 genes define distinct cell progenitors in the VZ, providing a beautiful example to see whether Purkinje cell and cerebellar nuclei neurons derive from common cell precursors.

The use of *in vivo* electroporation to conduct both gain- and loss-of-function studies of gene function in the cerebellum will contribute to the elucidation of the molecular components and gene networks involved in cerebellar histogenesis. *In vivo* electroporation can be employed to understand the role of individual transcription factors as well as unique combinations of them. The strategy of overexpressing

combinations of the transcription factors identified in this study can unravel the existence of gene regulatory networks acting at early neurogenesis. Furthermore, it can give key insights into the relationship between transcription factors and cell fate specification in the developing cerebellum.

Time-lapse video microscopy has proven to be a powerful tool to understanding shape changes and dynamics during animal morphogenesis. The possibility of expressing EGFP in a cell-specific manner by using BAC transgenic mice, together with state-of-the-art videomicroscopy will make it possible to register the complex cell population movements that shape the cerebellar tissue during histogenesis. The data coming from these proposed analyses will fill the gap in our current knowledge of the cell dynamics involved in the formation of the cerebellar nuclei and cerebellar cortex.

Homeodomain proteins define neural cell identity in the developing neural tube

Earlier studies have shown that LIM homeodomain proteins play a key role in the specification of distinct neural cell populations in the developing neural tube (Kania et al., 2000; Kania and Jessell, 2003; Thaler et al., 2004). In the dorsal half of the developing spinal cord, distinct neuronal populations can be distinguished by unique combinations of transcription factors (Lee and Jessell, 1999). The most dorsal spinal interneurons, dI1 (D1) neurons, are delineated by Math1, Lhx2/9 and Brn3a expression; dI2 (D3A) neurons are defined by Ngn1, Ngn2, Lhx1/5, Brn3a and Foxd3; dI3 (D2) neurons express Isl1 and Brn3a (Gowan et al., 2001; Gross et al., 2002.; Lee and Jessell, 1999). Notably, the migratory journey of dI1 (Lhx2/9^{+}, Math1^{+}) interneurons is reminiscent of the circumferential migratory pathway taken by cerebellar nuclei neurons (also Lhx2/9^{+} but Math1-), with precursors migrating out of the VZ to the superficial mantle zone, then moving in the plane of the pial surface distally, to subsequently migrate inwardly as coalescing into distinct nuclei in a ventral position.

It has been shown that the program of motor neuron specification in the spinal cord involves the formation of hetero-complexes between LIM homeodomain proteins and members of the

bHLH transcription factor family (Lee and Pfaff, 2003). Although we have found that cerebellar nuclei neurons and Purkinje cells express unique combinations of LIM homeodomain proteins soon after cell cycle exit, so far, we have not found expression of bHLH transcription factors in these two cell populations. Precursors of granule cells require expression of the bHLH protein Math1 for their differentiation, however, among the Lhx genes we analyzed we did not find any with restricted expression in the EGL. Instead, we found that granule cell progenitors expressed the TALE transcription factor family member Meis1. We are currently investigating whether cerebellar cell specification proceeds through other mechanisms than the spinal cord.

TALE transcription factor family members Irx and Meis have been implicated in regulating morphogenesis of several embryonic structures, including the isthmic organizer, limbs, eyes and hindbrain (Bosse A, 1997; Choe et al., 2002; Lebel et al., 2003; Mercader et al., 1999; Waskiewicz et al., 2001; Zhang et al., 2002). A recent study has shown that Fgf8 modulates Irx2 activity via a MAP kinase cascade in the isthmus (Joyner et al., 2000; Matsumoto et al., 2004; Ye et al., 2001). Misexpression of both *Irx2* and *Fgf8a* induces ectopic cerebellum in the midbrain, suggesting a key role for Iroquois genes in the formation of the cerebellum. Whereas these results show that Irx2 is critical for cerebellar formation, our findings suggests that *Irx* genes

also function in cell differentiation events required for cerebellar histogenesis. Studies on Meis proteins functions indicate that the formation of multimeric complexes with Hox and Pbx transcription factor family members is critical for transcription activation of target genes both *in vitro* and *in vivo* (Jacobs et al., 1999; Vlachakis N, 2001). Retinoic acid (RA) induces the expression of Meis1 and Meis2 during limb development, an effect that is counteracted by Fgf (Mercader et al., 2000). It remains to be seen whether the Meis proteins expressed by precursors of the cerebellar neurons bind to Pbx proteins to regulate neuronal cell differentiation, and also whether RA and Fgfs modulate the action of Meis proteins within the cerebellum.

An interesting aspect of the present study is the correspondence between the patterns of transcription factor expression in neurons of the cerebellar nuclei, granule cells and Purkinje cells in the developing cerebellum of chicks and mice. These findings agree with the emerging results from studies on the evolution of the brain, where patterns of transcription factors mark particular brain regions and principal cell classes from the lowest vertebrates through mammals (Jarvis et al., 2005). In the lamprey, although Fgf8 is expressed in the isthmus, the rhombic lip remains open and a cerebellar cortex never forms. In jawed fish and amphibians (bullfrog), a primitive cortex of a single layer of granule cells and Purkinje cells is present. Ascending the phylogenetic

tree of vertebrates, the total number and diversity of different types of neurons in the cerebellum increases. Thus stellate cells, the oldest cerebellar interneuron, appear in more specialized fish, followed by the basket cells in reptiles and other fish. The murine cerebellum contains some 7-8 classes of interneurons. Although cerebellar nuclei are not apparent in fish, the number of cerebellar nuclei and diversity of their cell types increases from lizards and birds (1-2 nuclei) to mice (3 cerebellar nuclei) to man (4 cerebellar nuclei). Population matching in two parallel “loops” or circuits through the cerebellum in primates and cetaceans (whales and dolphins) illustrate this point. All of the components of the circuit from the inferior olive to the lateral zone of the cerebellar cortex and then down through the lateral cerebellar nucleus (the dentate nucleus) are larger in primates than cetaceans, whereas the reverse is true of the circuit that loops through the main accessory olive of the brainstem through the intermediate zone of the cerebellar cortex and the posterior interpositus cerebellar nucleus. It will be important to understand how specification of the principal classes of neurons in a region relate to the changes in the diversity of cell types, size of the region and population matching of the neural circuitry.

Cerebellar complexity appears to be driven, in large part, by increasing ratios of granule neurons to Purkinje cells, through coordinated control of the granule cell progenitor proliferation. In

mammals, although the number of Purkinje cells increases (200,000 in mice, 3,000,000 in cats and 15,000,000 in man), and the ratio of granule cells to Purkinje cells rises dramatically. In mouse, the ratio of granule cells to Purkinje cells is 200:1; in humans it is 3,000:1. These changes likely increase the ability to refine the time scale and directional coordination of motor control, sensory discrimination, and cognitive control of cerebellar functions. Regulators of the cell cycle in the aRL, a source of granule cells and neurons of brainstem nuclei, and the timing of specification of different classes of neurons are likely to provide fundamental insights on the evolution of cerebellar circuitry and its role in brain function.

Our findings are consistent with the emerging view that specific patterns of transcription factor expression govern the specification of neural progenitors. The cerebellum presents as especially interesting case, as the specification of the principal classes of neurons, shown here to relate to the expression of specific transcription factors, acts in concert with so-called “organizers” such as *Fgf8* and *Irx2* to pattern the cerebellar territory. It will be important to understand, on a molecular level, how genes that mark specific neural populations as well as those involved in the directed migrations and axon-targeting of cerebellar neurons are regulated by the isthmic organizer genetic network. Unraveling these complex interactions in the cerebellum -- “the little

cortex" -- will provide insights into the development of higher cortical regions of the brain, as the evolution of these higher structures occurred after the evolutionary emergence of the cerebellum.

Materials and Methods

Animals

C57BLJ6 and FVB/N mice were housed in the Rockefeller University's animal facility on a light/dark cycle of 12 h. Timed pregnancies were established by checking vaginal plugs every morning, with the day of the sperm impregnation being designated embryonic stage 0.5 (E0.5). Embryos were collected between stages E10-E16.5 from anaesthesized pregnant dams. Postnatal day 6 (P6) and adults were also analyzed. The generation of *Irx3*, *Math1*, *Meis1*, *Lhx1*, *Lhx5* and *Pcp2* BAC transgenic mouse lines carrying the Egfp gene reporter was done following procedures reported in (Gong et al., 2003). Fertilized White Leghorn (*Gallus gallus*) chicken eggs (Spafas, Connecticut, USA) were incubated at 37.5°C with a relative humidity of 90% in a circulated air incubator (G.Q.F. Manufacturing Co. Georgia, USA). The first day of incubation is designated as embryonic day 0 (e0). Embryos were collected between stages e4 (HH23)-e7 (HH31) (Hamburger and Hamilton, 1992).

Immunohistochemistry

Embryos were fixed by immersion in 4% paraformaldehyde in phosphate-buffered saline at 4°C. For immunostaining on postnatal and

adults brains, the animals were anaesthesized with Nembutal and fixed by transcardial perfusion of 4% paraformaldehyde/PBS. Cryostat sections were blocked for 1h in PBS containing 3-5%NGS and 0.1% Triton X-100. Incubation with primary antibodies was done at 4 °C overnight, followed by rinses in PBS and incubation with secondary antibodies for 1 hr at room temperature. Fluorescent-conjugated secondary antibodies were used to detect the primary antibodies (Jackson Immunoresearch, Pensylvania, USA and Molecular Probes, California, USA). The primary antibodies used in this study were: rabbit anti-Lhx1/5, rabbit anti-Lhx2/9 and rabbit anti-Irx3 (gift of T. Jessell, New York), rabbit anti-Meis1 and rabbit anti-Meis2 (gift of A. Buchberg, Philadelphia), mouse anti-Reelin (gift of A. Goffinet, Brussels), rabbit anti-BLBP (gift of N. Heintz, New York), mouse anti-RC2 (gift of M. Yamamoto, Tsukuba), mouse anti-Calbindin (Swant, Bellinzona, Switzerland), mouse anti-Tuj1 (Covance, California, USA), rabbit anti-EGFP (Molecular probes, California, USA) and sheep anti-EGFP (Biogenesis, New Hampshire, USA). Images were collected on a Zeiss fluorescence microscope using a SPOT camera (Diagnostic instruments Inc, Michigan, USA) or a Radiance 2100 confocal microscope (BioRad, California, USA).

RNA in situ hybridization

In situ hybridization on whole mount brains or tissue sections was performed as described in (Sanders et al., 2002). Briefly, embryos were fixed in 4% paraformaldehyde/PBS at 4 °C overnight. Samples were incubated in detergent solution and then post-fixed with 4% paraformaldehyde/PBS. RNA probes synthesized using digoxigenin labelled nucleotides (Roche, Indianapolis, USA) were diluted in hybridization solution (50% formamide, 5X SSC, 2% SDS, 500 μg/ml tRNA, 2% BBR, and 50 μg/ml heparin) and applied at 70 °C overnight. After hybridization, the samples were washed first in solution X (50% formamide, 2X SSC, and 1% SDS) and then with TBST (25 mM TrisHCl, pH:7.5, 136 mM NaCl, 2.68 KCl, and 1% Tween). Probes to cMeis1 (M. Torres), cIrx3 (T. Jessell), mMath1 (Q. Ma) were detected with alkaline phosphatase-conjugated anti-digoxigenin antibody. Color reactions were performed with the substrates for alkaline phosphatase 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and tetranitroblue tetrazolium chloride(TNBT).

BrdU staining

Pregnant mice were injected intraperitoneally with a solution of 5-bromo-2'-deoxyuridine (BrdU) in water between E10-E11.5 (Sigma;

50μg/g body weight). Embryos were collected between stages E11-E12.5. Cryostat sections were incubated in 2N HCl at 37°C for 30 min, rinsed in PBS and incubated in 0.1 M boric acid, pH 8.5 for 10 min. The sections were blocked for 1h in PBS with 10% normal goat serum and 0.1% Triton X-100, followed by incubation with anti-BrdU monoclonal antibody (Beckton-Dickinson) in 5% NGS at 4 °C overnight. For fluorescent detection we used Cy3 and FITC-conjugated secondary antibodies.

In vitro induction assays

E8.5 whole mouse embryos (6–10 somites) were explanted and cultured for 72 hrs. The mes/met region was dissected in L-15 medium (Gibco). The presumptive dorsal cerebellum was treated with beads previously incubated with activin B, BMP6, BMP7, noggin, follistatin or PBS. The whole embryo explants were set in the incubator at 37°C in medium containing 47.5% Dulbecco's minimum essential medium (Specialty Media), 47.5% F-12 Ham's nutrient mixture (Gibco), 5% rat serum (Gibco), 100 U/ml penicillin-streptomycin (Gibco), 2 mM glutamine (Gibco) and 3.3 mM glucose. After 72 hrs in culture the embryos were fixed and assayed for immunostaining with Lhx1/5, Lhx2/9 and Meis2 antibodies. Lhx1/5 was used as a marker for

precursors of Purkinje cells, and Lhx2/9 and Meis2 as markers of precursors of the cerebellar nuclei.

Generation of *Egfp*-BAC transgenic mice

The generation of BAC mouse lines for genes of interest involved the replacement of the endogenous mRNA and protein coding sequences by the one encoding the EGFP reporter gene (Gong et al, 2003). This methodology uses most of the 5' and 3' regulatory elements flanking the gene of interest, making it possible to visualize EGFP expression in a pattern that recapitulates that of the endogenous gene.

The pLD53.SC2 plasmid containing *Egfp* and a PolyA tail were used to modify BACs containing genes of interest. Modified BAC DNA was prepared as described, and transgenesis was performed as described previously (Gong et al., 2003). Gene expression in embryonic mice was examined after fixation in 4% paraformaldehyde by immersion (12h, 4°C) Serial sagittal sections were generated with a Leica cryostat, after which the sections were fixed in methanol and used immediately for confocal analysis or immunostained with antibodies against Egfp as described (Gong et al., 2003). Generation of the *Egfp*-BAC transgenic mice was carried out by staff of the Gensat Project of which MEH is a Co-PI.

In ovo electroporation

Eggs were windowed at E2 by standard techniques. Briefly, a sterile needle was inserted into the blunt end of the egg and 2.5 ml albumin was removed to drop the embryo from the surface of the egg shell. A circular window was prepared on the lateral side of the egg using a fine curved scissors. Avian Ringer's solution containing 10 u penicillin-streptomycin (Invitrogen) was added to rehydrate the embyro as necessary. Following manipulation the prepared window was sealed with polyethylene tape (Fisher Scientific), and the egg was returned to the incubator until collection.

For *in ovo* electroporation of select expression constructs, a protocol similar to that described by Agarwala et al. (2001) was followed. Embryos were electroporated between E2 and E3, HH 12-19. After the egg was windowed, the amniotic and vitelline membranes were teased apart overlying the site to be electroporated, the midbrain-hindbrain region of the embryo. A pulled borosilicate pipette (1.2 mm outer diameter, Sigma) with a tip diameter of approximately 30-50 μm was front-filled with the injection solution by application of vacuum. The injection solution contained 1 μg/μl of the selected DNA construct in 10 mM Tris, 1 mM EDTA (pH 7.5) containing 0.1% Fast Green (Sigma) for visual monitoring of the injection. Plasmid DNA was prepared using the Wizard Purefection Plasmid DNA Purification System (Promega) and

stored in aliquots at -20°C. The pipette was inserted through the neuroepithelium into the neural tube lumen and the DNA-containing solution was pressure injected until the cerebellar region to be transfected was completely filled by the tracking dye (Harvard Apparatus picopump PLI-90).

Two platinum L-shaped electodes, diameter of 250 μm, are separated by a distance of 2 mm with a commercially available holder (Genetronics). The electrode combination is connected to the BTX Electro Square Porator ECM 830 (Genetronics). Electrodes are positioned in order that the rostral hindbrain neural tube lies between the electrodes. The anode of the electrode pair is adjacent to the rhombic lip to promote the migration of the injected negatively charged DNA into cells of the rhombic lip. Subsequently, a train of square wave pulses is delivered to the embryo with the following parameters: 6 pulses, 8 - 10 volts, 50 ms pulse duration, 1 s interpulse interval.

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