2 Proneural Genes and Cerebellar Neurogenesis in the Ventricular Zone and Upper Rhombic Lip

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Abstract

The cerebellar primordium arises between embryonic days 8.5 and 9.5 from dorsal rhombomere 1, adjacent to the fourth ventricle. Cerebellar patterning requires the concerted action of several morphogens secreted by the rhombic lip and roof plate and leads to the formation of two main neurogenic centers, the upper rhombic lip and the ventricular zone, from which glutamatergic and GABAergic neurons arise, respectively. These territories contain gene expression microdomains that are partially overlapping and, among others, express proneural genes. This gene family is tightly conserved in evolution and encodes basic helix-loop-helix transcription factors implicated in many neurogenetic events, ranging from cell fate specification to terminal differentiation of a variety of neuronal types across the embryonic nervous system. The present chapter deals with the established or suggested roles of proneural genes in cerebellar neurogenesis. Of the proneural genes examined in this chapter, *Atoh1* plays a quintessential role in the specification and development of granule cells and other cerebellar glutamatergic neurons. Besides playing key roles at early stages in these early developmental events, *Atoh1* is a key player in the clonal expansion of GC progenitors of the external granule layer. *NeuroD*, formerly regarded as a proneural gene, acts as a master gene in granule cell differentiation, survival, and dendrite formation. *Ascl1* participates in GABA interneuron and cerebellar nuclei neuron generation and suppresses astrogliogenesis. Conversely, less is known to date about the role(s) of *Neurog1* and *Neurog2* in cerebellar neurogenesis, and a combination of loss- and gain-of-function studies is required to elucidate their contribution to cerebellar neurogenesis.

Keywords

Achaete/scute like 1 · *Amos* · *as-c* complex · *Ascl1* · *Ato* · *Atoh1* · *Atoh5* · *Atonal* · *Atonal homolog 1* · Basic helix-loop-helix · bHLH · *Biparous* · BMP · Bone morphogenetic protein · *Cato* · *Cerebelless* · *Drosophila* · *Drosophila melanogaster* · FGF8 · GABAergic neurogenesis · Genetic fate mapping · Gli2 · Granule cell clonal expansion · Granule cell development · *Lhx1* · *Lhx5* · *Lmx1a* · *Mash1* · *Math1* · Medulloblastoma · *NeuroD* · *Neurog1* · *Neurog2* · *Neurogenin 1* · *Neurogenin 2* · *Ngn1* · *Ngn2* · Notch · Notch signaling · NTZ · Nuclear transitory zone · Olig2 · Patched · Pax2 · Proneural gene · Proneural genes · *Ptf1a* · *sc* · SHH · *Smoothened* · Sonic hedgehog · Sox9 · Upper rhombic lip · URL · Ventricular zone · Vertebrate neurogenesis · VZ · WNT · *Xath1* · *Xath5* · *Xenopus laevis* · *XneuroD* · Zic1

Introduction

The mature cerebellum represents only 10% of the total brain volume but contains the majority (80–85%) of human neurons (reviewed in Herrup and Kuemerle 1997). It is the primary center of motor coordination, and it is essential for cognitive processing and sensory discrimination (Schmahmann 2004). In humans, the

cerebellum achieves its mature configuration only many months after birth, and for this reason, it is especially vulnerable to developmental abnormalities. Like the cerebrum, the cerebellum comprises an outer cortical structure, a layer of white matter, and a set of cerebellar nuclei (CN) beneath the white matter. The CN project efferent fibers to the thalamus, brainstem, and spinal cord (Paxinos 1994) mediating the fine control of motor movements and balance.

During mouse embryonic development, the cerebellum arises between embryonic days (E)8.5 and 9.5 from dorsal rhombomere 1 (r1), adjacent to the fourth ventricle, due to the mutual interactions between patterning genes, including *Gbx2*, *Otx2*, *Fgf8*, *Wnt1*, *En1*, *En2*, *Pax2*, *Pax5*, and others (reviewed in Liu and Joyner 2001). Cerebellar development requires a contribution from the posterior mesencephalon (Martinez and Alvarado-Mallart 1987) and the alar plate of rhombomere 2 (Marin and Puelles 1995). Normal patterning of the cerebellar anlage (Fig. 1a, b) depends on the formation and function of the isthmic organizer, a signaling center secreting the morphogens fibroblast growth factor 8 (FGF8) and WNT1, that defines the cerebellar territory along the anterior–posterior axis of the central nervous system (Martinez and Alvarado-Mallart 1987; Marin and Puelles 1994; Crossley et al. 1996; Wassarman et al. 1997; Martinez et al. 1999) and entails a fundamental contribution by the roof plate, secreting WNT and bone morphogenetic protein (BMP) ligands (Alder et al. 1999; Chizhikov and Millen 2004; Chizhikov et al. 2006). The patterning of the cerebellar primordium leads to the formation of two main germinal centers that will give rise to the multitude of neuronal types and subtypes observed in the mature cerebellum. These germinal regions, called ventricular zone (VZ) and upper rhombic lip (URL), contain gene expression microdomains that are partially overlapping and that will regulate the genesis of neuronal precursors fated to adopt GABAergic and glutamatergic phenotypes, respectively. Among other genes expressed in these microdomains are four proneural genes, namely, *neurogenin 1 and neurogenin 2* (*Neurog1/Ngn1*, *Neurog2/Ngn2*) *and achaete/scute like 1* (*Ascl1/Mash1*) in the VZ, as well as *Atonal homolog 1* (*Atoh1/Math1*) in the URL (Fig. 1c).

Proneural Genes in *Drosophila melanogaster* Development

The defining event of neurogenesis is the switch from uncommitted, cycling progenitor cells to committed neuronal precursors. Nervous system development in *Drosophila melanogaster* has served as a paradigm for the discovery and dissection of neurogenetic processes and their regulation, providing a conceptual framework for the understanding of mammalian neurogenesis. In *Drosophila*, the entire nervous system arises from neuroectodermal cells, which delaminate from the surface epithelium and migrate into the interior of the embryo to form neural precursor cells or neuroblasts. The first step of neural fate determination in the *Drosophila* nervous system is the singling-out of neuroblasts from the neuroectodermal epithelium. Prior to such cell-selection process, all the ectodermal precursors share an equivalent potentiality to become either neuroblasts or epidermoblasts. The choice between these alternative fates was proven to rely on the expression of a small group of

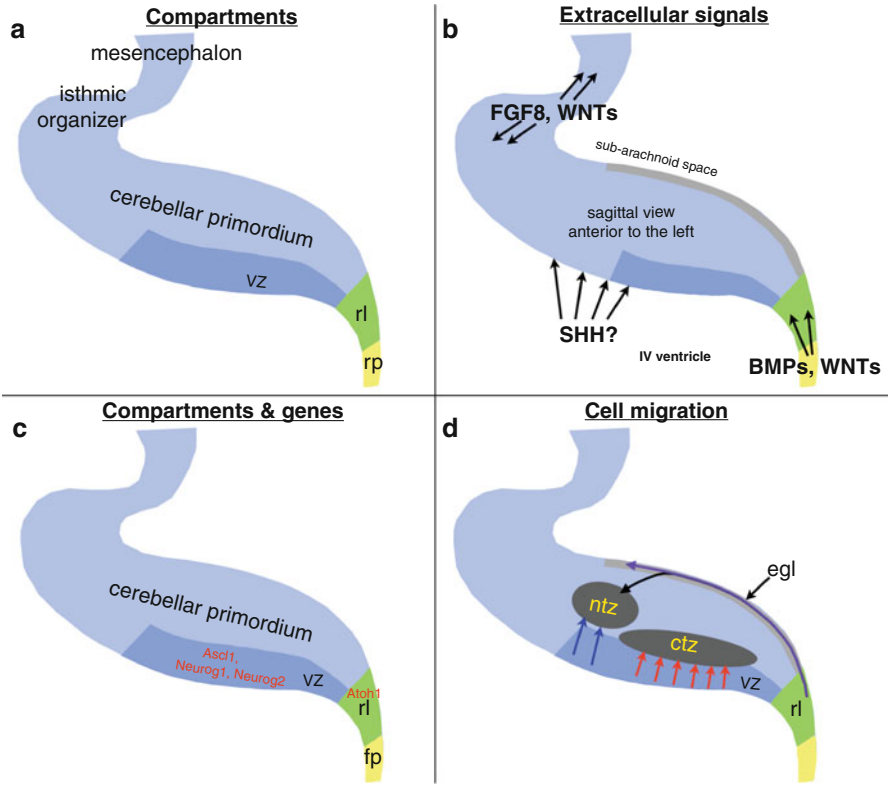

Fig. 1 Schematic of a cerebellar primordium with respect to patterning (**a**, **b**), neurogenesis (gene names in red boldface, **c**), and neuronal migration. See text for details. In (**d**) (highly simplified illustration), vertical upward arrows indicate radially migrating ventricular zone progenitors. Curved arrows indicate granule cell precursors (purple) and cerebellar neuron precursors (black). *ctz* cortical transitory zone, *egl* external granule layer, *ntz* nuclear transitory zone, *rl* upper rhombic lip (abbreviated as URL in the text); *rp* roof plate, *vz* ventricular zone

transcription factors, belonging to the basic helix-loop-helix (bHLH) family, which instruct neuroectodermal cells to take up the fate of neural precursors – from which they were termed proneural genes (reviewed in Jan and Jan 1994).

Several expression studies have shown that a combination of upstream regulatory genes acts on the promoter regions of proneural genes to induce their expression within the so-called proneural clusters, i.e., regularly spaced patches of cells, all of which initially share an equivalent potential to become neural progenitors (Skeath and Doe 1996). Progressively, through a cell-cell interaction process called lateral inhibition, clustered progenitors start competing one another. As a result of this competition, the expression of proneural genes becomes restricted to one single prevailing cell, which will delaminate to form the neuroblast and will maintain and further upregulate proneural gene expression. Conversely and simultaneously, the remaining progenitor cells within the cluster receive inhibitory signals from the

predominant neural precursor cell, and they partially or completely downregulate proneural gene expression, thereby acquiring the alternative fate of epidermoblasts (Artavanis-Tsakonas et al. 1999). Finally, singled-out neural progenitors undergo a stereotyped pattern of cell division producing a fixed number of daughter cells, which will terminally differentiate into the distinct cell types characteristic of the mature fly nervous system: neurons and associated support cells of the sensory organs (Jan and Jan 1994).

The final cell type that a given neuron is fated to adopt is decided in a hierarchical, stepwise fashion. At each step, the neuroblast restricts its developmental potential along specific neuronal lineages, undergoing binary or multiple cell fate choices at determined phenotypic branch points of the cell-type specification process. As previously mentioned, a striking observation, first made in *Drosophila*, is the fact that the expression of individual proneural genes is restricted to different neuronal lineages. Indeed, genes belonging to the *as-c* complex were shown to be specifically involved in the determination of neuroblasts in external sense organs; *ato* provides the competence to form chordotonal organs (a type of internal sense organs) and photoreceptors; and *amos* is employed in the formation of multidendritic neurons and olfactory sensilla.

Such observations raised the possibility that proneural genes might be involved not only in triggering a "generic" program of neural determination (epidermis versus neuroblast) but also in the subsequent specification of a given neural identity (e.g., multidendritic neurons versus chordotonal organs). Support to this hypothesis came from gain-of-function experiments: the ectopic expression of *as-c* induces the formation of ectopic external sense organs, while the forced expression of *amos* leads to ectopic multidendritic neurons and olfactory sensilla (Rodriguez 1990; Chien 1996; Parras 1996; Goulding et al. 2000).

Drosophila proneural genes had been initially subdivided into two classes, based on their function: (i) *determination* genes, like *ac*, *sc*, or *amos* – dominantly expressed before any sign of neuronal differentiation and acting in the sorting out of neural progenitors from the neuroectoderm – and (ii) *differentiation* genes, like *cato* and *biparous*, expressed after the selection of neural precursors and involved in the process of neuronal differentiation per se. The sequential activation of these two classes of proneural genes – in a regulatory cascade in which early expressed determination genes induce downstream effectors of differentiation – was in agreement with the stepwise progression of undifferentiated progenitors toward differentiated neural cells (Campuzano and Modolell 1992). Indeed, a classical concept of experimental embryology is that the transition from an undifferentiated to a fully differentiated cell comprises two steps: cell specification/determination and cell differentiation.

Nevertheless, such functional categorization of proneural genes turned out not to be completely exhaustive nor completely explanatory, as many proneural genes, like *atonal*, were proven to exert both functions or either, i.e., to trigger a generic program of neural development and/or to induce neuronal lineage or subtype-specific programs, depending primarily on the temporal- and regional-specific cues active throughout their expression window. Furthermore, an individual bHLH

protein could be required for several different cell types at different times or locations during development. Factors affecting the outcome of proneural gene function include the *context* (cellular and genetic) and *timing* of their expression. More sophisticated criteria were therefore required to assess proneural function in neural development in order to explain such functional complexity. Placing proneural genes in temporal and, whenever possible, epistatic cascades has been a major goal in the past few years. Mapping these cascades onto precise stages and cellular events of the neuronal differentiation pathway remains an open challenge.

The Roles of Proneural Genes in Vertebrate Neurogenesis

Cell fate decisions in the development of the vertebrate and invertebrate nervous systems are controlled by remarkably similar mechanisms. Among other ontogenetic programs, the coupling of neural determination and lineage/cell-type specification, by means of the combined action of proneural genes and positional identity determinants, is a mechanism strikingly conserved in evolution. Unsurprisingly, vertebrate genomes were proven to contain several orthologs of *Drosophila* proneural genes (Ledent and Vervoort 2001; Simionato et al. 2007). Based on the homology to their *Drosophila* counterparts, such genes have been isolated, cloned, and shown to play a pivotal role in vertebrate neurogenesis (Ghysen and Dambly-Chaudiere 1988; Lee 1997). As in *Drosophila*, vertebrate proneural genes commit cycling progenitor cells to a neuronal fate, which involves activation of Notch signaling and the induction of cell-cycle exit. As in *Drosophila* development, vertebrate proneural genes act in regulatory cascades, with early expressed genes inducing fate specification of neural progenitors and later-expressed genes regulating terminal differentiation (Cau et al. 1997; Lee 1997).

In terms of sequence conservation in the bHLH domain, the mouse atonal homologs *Atoh1* and *Atoh5* are the genes most closely related to *Drosophila atonal*. Loss-of-function studies in the mouse have shown that *Atoh1* is necessary for the generation of cerebellar granule neurons (see further) and for the development of the sensory epithelium of the inner ear (Ben-Arie et al. 1997; Chen et al. 2002), while *Atoh5* is essential for retinal ganglion cell production (Wang 2001). Close members of the ato family were also found in *Xenopus laevis* – *Xath1* and *Xath5* – where they have been shown to induce a neural fate (Kanekar et al. 1997; Kim 1997).

In *Xenopus laevis*, overexpression of *Neurog1* leads to a massive ectopic formation of *XneuroD*-positive neurons (Ma et al. 1996). Incidentally, *NeuroD*, a gene initially labeled as a proneural gene thanks to its homology to other proneural factors and to its ability to convert presumptive dorsal epidermis into neurons in *Xenopus laevis* (Lee et al. 1995), is in fact involved in later stages of neuronal differentiation in vertebrates. Again, in addition to their generic neurogenetic role, numerous lines of evidence indicate that proneural genes also specify neuronal subtype identity. For instance, in the neocortex, the proneural genes *Neurog1* and *Neurog2* are absolutely required to specify the identities of early born, deep layer neurons, whereas they are dispensable for later stages of neuronal fate specification (Schuurmans et al. 2004).

In the developing spinal neural tube, *Atoh1* on one hand and *Neurog1* and *Ascl1* on the other are expressed in nonoverlapping patterns, and cross-inhibition occurs between the two sets of genes (Gowan et al. 2001). Furthermore, constitutive or ectopic expression of neurogenins, and other atonal homologs, yields specific neuronal subtypes in vivo (Blader et al. 1997; Kanekar et al. 1997; Olson et al. 1998; Perez et al. 1999; Gowan et al. 2001). *Neurog1* or *Neurog2* single-mutant mice lack complementary sets of cranial sensory ganglia, and *Neurog1/2* double mutants lack, in addition, spinal sensory ganglia and a large fraction of ventral spinal cord neurons (Fode et al. 1998; Ma et al. 1998, 1999). Likewise, loss-of-function and gain-of-function analyses have shown that *Ascl1* contributes to the specification of neuronal subtype identity (Fode et al. 2000) and is both required and sufficient to specify ventral telencephalic fates. Further evidence indicates that *Ascl1* acts cooperatively in sympathetic ganglia as an instructive determinant of cell fate to induce the noradrenergic phenotype (Goridis and Brunet 1999). *Ascl1* has also been implicated in the differentiation of early-born (but not late-born) neurons in the striatum, indicating that it regulates both progenitor cell behavior and neuronal fate specification in a temporally defined manner (Casarosa et al. 1999; Yun et al. 2002).

The present chapter deals with the established or presumptive roles of proneural genes in the context of neuronal type specification, determination, differentiation, and clonal expansion at various stages during cerebellar neurogenesis. This chapter will provide a review of the literature about the roles played in that context by four established proneural genes and by one of their targets, *NeuroD*, formerly considered a proneural gene. Emphasis will be placed on the evidence accrued from the analysis of wild-type and transgenic mice.

Atoh1: The Master Gene in Granule Cell Development

More than a century ago, the URL was recognized as the origin of the most numerous neuronal population in the CNS, the cerebellar granule cells (GCs), that first migrate tangentially (Fig. 1d, purple arrow) and then radially during embryonic and postnatal development, respectively (Cajal 1889; Schaper 1897). During postnatal proliferation in the external granule layer (EGL), GC precursors maintain *Atoh1* expression until they radially migrate inward to form the internal granule layer (IGL) (Hatten and Heintz 1995; Helms et al. 2001). *Atoh1* is the murine homolog of the *Drosophila* proneural gene *atonal*, as shown by sequence similarity and functional conservation in evolution (Ben-Arie et al. 2000). It encodes the homonymous bHLH transcription factor and is expressed starting at E9.5 in the dorsal neural tube, from rhombomere 1 to the tail (Akazawa et al. 1995). In the URL, *Atoh1* is expressed in progenitors distinct from *Lmx1a*+ roof plate cells (Chizhikov et al. 2006). *Atoh1* plays key roles in the development of all glutamatergic neurons of the cerebellum. The cerebellum of *Atoh1* $^{-/-}$ mice is reduced in size and displays no foliation. In *Atoh1* null embryos, unlike at earlier stages (Chizhikov et al. 2006), BrdU incorporation is strikingly reduced in the rhombic lip at E14.5, and the EGL fails to form, resulting in the complete absence of cerebellar GCs (Ben-Arie et al.

1997; Bermingham et al. 2001). Instead, Purkinje cells (PCs), which form normally in the cerebellar VZ, migrate into the outermost part of the cerebellum and form a rudimentary PC layer, although some PCs fail to migrate toward the cortex and are retained in deep regions of the cerebellar primordium.

Balanced levels of *Atoh1* expression are essential for the correct timing of GC precursor differentiation. As mentioned, *Atoh1*-null mice have a small, poorly foliated cerebellum devoid of GCs. However, while *Atoh1* overexpression leads to the upregulation of early differentiation markers (e.g., *NeuroD*, *Dcx*), it is insufficient to promote a complete differentiation of GC precursors (Helms et al. 2001). This paradox can be explained by the fact that, physiologically, *Atoh1* is downregulated by its own protein product through a negative regulatory feedback loop (Gazit et al. 2004), and by other factors such as Notch intracellular domain, and the zinc-finger transcription factor Zic1 at the onset of GC precursor migration from the EGL to the IGL (Ebert et al. 2003). Likewise, in vitro experiments demonstrated that bone morphogenetic protein (BMP) signaling activation leads to the posttranslational inactivation of Atoh1, which is targeted to the proteasome for degradation (Zhao et al. 2008).

Atoh1 Plays a Key Role in Granule Cell Clonal Expansion

Peak cerebellar growth occurs relatively late compared to the rest of the brain, driven primarily by proliferation of EGL cells. In the mouse, starting at the end of embryonic development (around E16.5), GC precursors resident in the EGL start undergoing an impressive wave of clonal expansion, before differentiating and undertaking their radial migration into the IGL. The highest growth rate is recorded during the first 2 weeks of postnatal life, while in humans, the corresponding proliferative peak occurs in utero during the third trimester, although EGL remnants can persist for up to a year after birth (Abraham et al. 2001). This prolonged ontogenetic period makes the cerebellum susceptible to developmental aberrations and tumor formation. Medulloblastoma (MB), the most common brain tumor of childhood, has been studied by histological means and, more recently, by molecular analysis (Eberhart et al. 2000; Thompson et al. 2006). Physiologically, the clonal expansion phase is promoted non-cell-autonomously by PCs, as first shown by John Oberdick and coworkers through transgenic expression of the diphtheria toxin in these neurons, leading to the virtual abolition of GC precursor proliferation in the EGL (Smeyne et al. 1995). This non-cell-autonomous effect is driven by the secreted morphogen and mitogen sonic hedgehog (SHH), as shown by Matthew Scott's group and by others (Dahmane and Ruiz-i-Altaba 1999; Wallace 1999; Wechsler-Reya and Scott 1999). In fact, in conditional knockout mice in which SHH is specifically removed from PCs, MacMahon and coworkers (Lewis et al. 2004) observed a drastic reduction of GC precursors expansion. Accordingly, heterozygous mutants for the inhibitory SHH receptor patched, in which SHH signaling (reviewed in Villavicencio et al. 2000) is upregulated in a ligand-independent fashion, are prone to the development of medulloblastoma (MB)-like tumors. Among the MB

subtypes that have been characterized in patients, the desmoplastic variant seems to be derived from *Atoh1*+ GC precursors in the EGL (Pomeroy et al. 2002; Salsano et al. 2004). Collectively, this evidence prompted questions as to whether *Atoh1* plays any role in GC precursor proliferation.

Zoghbi and coworkers addressed this point in a paper published in 2009 (Flora et al. 2009) showing that *Atoh1* is required for the peri- and postnatal expansion of GC progenitors. To investigate the molecular effects of *Atoh1* deletion, they isolated GC progenitors from *Atoh1*$^{flox/flox}$ P5 mice and infected them with adenoviruses expressing either the green fluorescent protein (GFP) or the Cre recombinase gene and cultured transduced cells in the presence of SHH to evaluate their proliferative status. Their results showed that *Atoh1* deletion led to a sharp decrease in cell proliferation, suggesting that, in those cells, the response to SHH is largely dependent on *Atoh1*. Through various approaches, they showed that *Atoh1* mediates this response by transcriptionally upregulating the expression of *Gli2*, a critical mediator of SHH signaling (reviewed in Villavicencio et al. 2000). Next, they asked whether its expression might be required for the genesis of MBs induced by constitutive activation of the SHH pathway. To address this point, they crossed mice harboring a conditional deletion of *Atoh1* with tamoxifen-inducible Cre deleters expressing a constitutively active form of the SHH co-receptor smoothened. While, in the presence of *Atoh1*, *smoothened* overexpression caused hyperplasia of the EGL, this effect was drastically quenched in mice carrying a homozygous *Atoh1* deletion.

Other Glutamatergic Neurons Derive from *Atoh1*+ Progenitors

Some *Atoh1*+ cells migrate from the RL to the nuclear transitory zone (NTZ) (Jensen et al. 2004) (black downturned arrow in Fig. 1d), a transitory cell cluster that will give rise to the cerebellar nuclei (CN). A paper published in 2005 by Gord Fishell's group (Machold and Fishell 2005) clearly revealed that GCs are not the only glutamatergic lineage derived from *Atoh1*+ progenitors of the URL. This work was conducted taking advantage of a mouse line expressing a tamoxifen-inducible form of Cre recombinase (CreER^{T2}) under transcriptional control of the *Atoh1* promoter. This approach, dubbed genetic inducible fate mapping (Joyner and Zervas 2006), allowed the authors to tag several waves of neuronal progenitors born at successive stages of development, determining their final fate and location in the adult brainstem and cerebellum, shedding light on the spatiotemporal regulation of cerebellar glutamatergic neurogenesis. The results of this study clearly demonstrated that, prior to E12.5, *Atoh1* is transiently expressed in cohorts of cerebellar rhombic-lip neural precursors that populate the ventral hindbrain and deep cerebellar nuclei in the adult. Starting at ~E12.5, *Atoh1*+ progenitors start giving rise mostly to granule cells that will populate the anterior lobe; finally, *Atoh1*+ progenitors born at later stages until E16.5 will progressively populate the entire EGL and, after inward migration, the IGL of all cerebellar lobules.

A parallel study, conducted by Zoghbi and colleagues (Wang et al. 2005), took advantage of an *Atoh1*$^{LacZ/+}$ knock-in line to analyze the migration and fate of *Atoh1*

+ progenitors and corroborated the findings of Machold and Fishell. In addition, this study, using a *Atoh1*$^{LacZ/-}$ null mutant, demonstrated that *Atoh1* is essential for the establishment of the NTZ first and eventually for the formation of the glutamatergic component of CNs. Thus, *Atoh1* is required to support the development of both GCs and the glutamatergic CN neurons. The role of *Atoh1* in precerebellar nuclei development is discussed elsewhere (Machold and Fishell 2005; Wang et al. 2005) and is beyond the scope of the present chapter.

Late *Atoh1*+ Progenitors in the URL Give Rise to Unipolar Brush Cells

Unipolar brush cells (UBCs) are the other glutamatergic neurons located in the adult IGL. They were first described for their morphology: they feature a single brush-like dendritic ending (Harris et al. 1993; Mugnaini and Floris 1994) and project their axons to GCs and to other UBCs (Nunzi et al. 2001). In 2006, Hevner and colleagues showed that UBCs also originate from the *Atoh1*+ URL that produces GCs, except that they do so later in development (Englund et al. 2006). UBC precursors are born in the URL between E15.5 and E17.5 and migrate into the prospective white matter during late embryonic and early postnatal development. Interestingly, while *Atoh1* $^{-/-}$ mice show the complete loss of GC precursors, UBCs were severely reduced but not completely depleted in the same mutants.

In the context of cerebellar neurogenesis, *Atoh1* expression in the URL plays a quintessential role in the specification and development of GC precursors and of progenitors of other neurons, namely, glutamatergic ones populating the CN, besides several ventral hindbrain nuclei. While *Atoh1* is expressed in UBC progenitors, it is not strictly required for the determination of this specific cell fate. Besides playing key roles at early stages in these early developmental events, *Atoh1* is a key player in the clonal expansion of GC progenitors in the EGL at the end of their tangential migration from the URL (Fig. 1d).

NeuroD: A "Nearly Proneural" Gene with Key Roles in Cerebellar Development

NeuroD is a bHLH transcription factor originally studied in *Xenopus laevis* for its ability to convert embryonic epithelial cells into differentiated neurons and for its role in promoting cell cycle exit and neuronal differentiation (Lee et al. 1995). A loss-of-function approach was used to study *NeuroD* functions during brain development (Miyata et al. 1999). *NeuroD* null mice died shortly after birth due to diabetes. In the study conducted by Miyata et al., the authors rescued this phenotype introducing a transgene that encodes the mouse *NeuroD* gene under control of the insulin promoter. The rescued null mice featured an ataxic gait, walked around incessantly, and failed to balance themselves. A histological analysis performed at P30 revealed a severe reduction of cerebellar GCs in the posterior lobules (VI–X), whereas a small population of GCs survived in the anterior cerebellum. Secondary to

GC depletion, PCs of the posterior cerebellum failed to arrange into a proper monolayer. However, PC development appeared normal in terms of PC-specific marker expression. At birth, *NeuroD* is expressed in the inner layer of the EGL where post-mitotic granule cells reside and in the post-migratory GCs located in the IGL. In null mice, at birth, Miyata et al. found an increased rate of cell death in the inner half of the posterior EGL, which harbors post-mitotic precursors. GC death continued until P30, indicating a degeneration of the surviving GCs of the IGL. The authors concluded that *NeuroD* regulates a transcriptional cascade of genes that are essential for the differentiation and survival of post-mitotic cerebellar GCs.

Another study conducted by Azad Bonni's group at Harvard University (Gaudilliere et al. 2004) revealed a novel role for *NeuroD* in regulating GC neuron dendritic morphogenesis. They demonstrated that the knockdown of NeuroD expression by RNA interference in both primary cerebellar GC culture and organotypic cerebellar slices resulted in a profound alteration of dendritic morphogenesis, while it had no effect on axonal growth. Moreover, they demonstrated that neuronal activity leads to the phosphorylation of *NeuroD* by the protein kinase CAMKII. This event activates a downstream intracellular signaling pathway that regulates dendritogenesis in cerebellar granule neurons. Additional studies will be necessary to fully dissect the transcriptional machinery downstream of *NeuroD* responsible for the growth and maintenance of granule cell neuron dendrites.

The above findings demonstrate that *NeuroD* acts as a master gene in the context of cell-intrinsic transcriptional mechanisms that guide GC differentiation, survival, and dendrite formation. It is worth mentioning that *NeuroD* is not only expressed in the progeny of URL progenitors. Expression of this gene is clearly detectable in the cerebellar cortical transitory zone (CTZ), a post-mitotic compartment hosting PC- and other GABAergic precursors (Croci and Consalez unpublished observation; Lee et al. 2000).

Ascl1 in Ventricular Zone Neurogenesis

Ptf1a Is a Master Gene of Cerebellar GABAergic Neurogenesis

Although the cerebellum contains a relatively small variety of neurons, the molecular machinery governing neuronal generation and/or subtype specification is still poorly understood. In 2005, Hoshino et al. (2005) published the characterization of a novel mutant mouse, *cerebelless*, which lacks the entire cerebellar cortex but survives up to adult stages. The analysis of its phenotype, and the characterization of the underlying gene mutation, clarified that *Ptf1a* (pancreas transcription factor 1a), which encodes a bHLH transcription factor, is required for generating the cerebellar GABAergic compartment. *Atoh1* and *Ptf1a* participate in regionalizing the cerebellar neuroepithelium and define two distinct areas, the VZ (*Ptf1a*) and the URL (*Atoh1*), which generate GABAergic and glutamatergic neurons, respectively (Hoshino et al. 2005; Pascual et al. 2007). Although *Ptf1a* is not a proneural gene,

the expression of three proneural genes (*Ascl1*, *Neurog1*, and *Neurog2*) overlaps with that of *Ptf1* in the VZ, warranting this brief foreword.

Ascl1 Labels the Cerebellar GABAergic Lineage

While many studies have investigated the distribution and roles of *Atoh1* in the cerebellar glutamatergic lineage, fewer studies have been devoted to analyzing GABAergic precursors born in the cerebellar VZ and to dissecting the roles of proneural genes in this context. In 2008, Zordan et al. (2008) published a systematic descriptive analysis of proneural gene expression at early stages of mouse cerebellar development. The results of this study established that at the onset of cerebellar neurogenesis, starting at about E11, the *Ascl1* transcript becomes detectable by in situ hybridization in the VZ and presumptive NTZ. A similar distribution is observed at later stages, with the *Ascl1* transcript occupying the entire thickness of the *Ptf1a*+ VZ all the way to its apical (ventricular) margin. Accordingly, the territories occupied by *Ascl1* and *Atoh1* are clearly complementary. The *Ascl1* transcript remains confined to the VZ until E13.5.

An additional study by Jane Johnson and coworkers (Kim et al. 2008), published 1 month later, reported the results of genetic fate mapping performed using two transgenic *Ascl1-Cre* lines, one of which expressed a tamoxifen-inducible Cre recombinase, CreER™ (Helms et al. 2005; Battiste et al. 2007), and two Cre-inducible reporter lines (Soriano 1999; Srinivas et al. 2001). The evidence produced in this elegant lineage analysis was in full agreement with the conclusions drawn by Zordan et al.: in particular, *Ascl1*+ progenitors are initially (E12.5) restricted to the cerebellar VZ and excluded both from the post-mitotic CTZ and from the rhombic-lip migratory stream. The locations of *Ascl1*+ and *Atoh1*+ progenitors are mutually exclusive, whereas a high degree of overlap was shown between *Ptf1a*+ and *Ascl1*+ progenitors, revealing that *Ascl1* labels GABAergic neuronal progenitors. However, at later stages (E17.5), *Ascl1*+ progenitors were no longer confined to the VZ but were detected in a scattered pattern throughout the cerebellar primordium. Some of these cells coexpressed Ascl1 and Olig2, which at this stage decorates oligodendrocytes. In addition, the fate mapping analysis contained in this study revealed that *Ascl1*+ progenitors born either before E11.5 or after E13.5 give rise to the GABAergic components of the CN, whereas progenitors born between E11.5 and E13.5 are mainly fated to become PCs. Finally, when *Ascl1*+ progenitors are tagged by tamoxifen administration after E16, some of them acquire an oligodendroglial identity and localize in the prospective white matter. In the same study, no colocalization was ever scored between *Ascl1*+ progenitors and astrocyte-specific markers.

Finally, a study authored by Marion Wassef and collaborators (Grimaldi et al. 2009) further refined the analysis of the role of *Ascl1* in cerebellar neurogenesis, incorporating an description of the effects of *Ascl1* gene disruption and overexpression. Regarding the distribution of *Ascl1*+ progenitors, Grimaldi et al. established that these precursors progressively delaminate out of the VZ to settle

in prospective white matter first and cerebellar cortex next. By studying an *Ascl1-GFP* transgenic mouse, they demonstrated that *Ascl1*+ progenitors give rise to interneurons positive for Pax2 and to oligodendrocyte precursors positive for Olig2. Conversely, glutamatergic neurons as well as astrocytes and Bergmann glia cells never expressed GFP.

To clarify the role of *Ascl1* in the generation of different cerebellar cell types, the authors analyzed *Ascl1* null mice at E18.5. The loss of *Ascl1* led to a dramatic reduction of *Pax2*+ and *Olig2*+ precursors, whereas astrocytic precursors, labeled by Sox9, were moderately increased in number. No major change was detected in PC development. Furthermore, to circumvent the perinatal lethality of *Ascl1* null mice and study the effect of *Ascl1* at later stages, the authors transplanted solid grafts obtained from E15.5 mutant- and wild-type cerebella into the cerebral cortex of newborn recipients. After 2 months, they analyzed the grafts, and, consistent with previous results, they found that they contained a reduced number of parvalbumin+ interneurons but a normal number of PCs.

Finally, the authors resorted to a gain-of-function approach (in vivo electroporation of a GFP plasmid, stage E14.5) to determine whether oligodendrocytes and Pax2+ interneurons are lineally related in the cerebellum. After electroporation, cerebella were dissected and cultured for 6 days in vitro. By comparing the results of intraventricular vs. parenchymal electroporations, they concluded that most *Ascl1* + oligodendrocytes do not originate from the cerebellar VZ. In addition, they electroporated an *Ascl1-GFP* vector into the cerebellar VZ. This led to an increased number of Pax2+ interneurons, to a reduced number of Olig2+ oligodendrocyte precursors, and to a complete absence of astroglial cells. This prompted them to conclude that *Ascl1* overexpression pushes progenitors toward the Pax2 interneuron fate while suppressing the astrocytic fate.

Taken together, the above evidence suggests that, in cerebellar neurogenesis, *Ascl1* contributes to the GABAergic pool. It participates in GABA interneuron and CN neuron generation and in PC development. However, it is not required for PC specification, suggesting that, in this process, it may either be irrelevant or act redundantly with other VZ-specific proneural genes. When overexpressed at E14.5, *Ascl1* promotes the GABA interneuron phenotype, suppressing the astrocytic fate.

Neurogenins in Cerebellar GABAergic Development

Neurog1 and *Neurog2* Are Expressed in the *Ptf1a*+ Ventricular Neuroepithelium

As shown by Zordan et al. (2008), the *Neurog2* transcript is first observed around E11 in CN neuron progenitors of the cerebellar primordium, whereas *Neurog1* appears 1 day later, in a rostral region located between the isthmic organizer, labeled by *Fgf8*, and the territory marked by *Ascl1*. At E12.5, both *Neurog1* (see also Salsano et al. 2007) and *Neurog2* are present in the VZ but with a few differences

in distribution: in the anterior cerebellum, *Neurog1* is expressed at high levels in a region close to the midline, whereas *Neurog2* is detected mostly in the lateral VZ. In posterior territories, the expression patterns of the two proneural genes overlap completely. *Neurog1* and *Neurog2* are adjacent and partially overlap with post-mitotic domains labeled by *Lhx1* and *Lhx5,* two genes that control PC differentiation (Zhao et al. 2007). This suggests that *Neurog1* and *Neurog2* are upregulated in progenitors that are undertaking the last cycle of cell division, to become post-mitotic PC precursors. At E13.5 the differential anterior boundaries of *Neurog1* and *Neurog2* are maintained, although the transcript levels of both genes are down-regulated. The authors conclude that *Neurog1* and *Neurog2* are mainly expressed in the cerebellar germinal epithelium that gives rise to GABAergic progenitors, while they are completely absent from the URL, the source of all glutamatergic cerebellar progenitors. Moreover, their expression patterns are similar but not totally overlapping, suggesting that they may contribute to the diversity of cerebellar GABA neurons and, possibly, PC subtypes.

Neurog1 Is Expressed in Cerebellar GABAergic Interneuron Progenitors

In 2009, Doughty and coworkers published a lineage analysis in which, by using transgenic fate mapping, they described the mature cerebellar neurons deriving from *Neurog1*-positive cell fates in the developing mouse cerebellum (Lundell et al. 2009) and extended their analysis of *Neurog1* expression to include late embryonic and postnatal cerebellar development. At E14–E20, *Neurog1* is present in *Ptf1a*+ neurons, but it is excluded from the URL and EGL. Moreover, at P7, it colocalizes with *Ptf1a* and BrdU in the cerebellar white matter. This suggests that *Neurog1* is expressed in early GABAergic interneuron precursors that, shortly after birth, migrate from the white matter to reach their destination in the cortex. To test their hypothesis, the authors used two artificial chromosome (BAC)-reporter mice imported from the NIH GENSAT consortium (Rockefeller University, New York) to study short-term and long-term *Neurog1*-positive cell fates. The first to be characterized was the *Neurog1-EGFP* transgenic mouse line, previously used to map short-term cell fates in the developing thalamus (Vue et al. 2007). Their results supported the hypothesis that *Neurog1* is expressed in *Pax2*+ interneuron progenitors. Surprisingly, they could not reveal any fluorescence in PC neurons. A recent study by Jane Johnson and coworkers, by genetic inducible fate mapping, confirmed the notion that, in the cerebellar primordium, the *Neurog1*+ lineage gives rise to PCs and to GABAergic interneurons (Kim et al. 2011). This notion was further confirmed in fine detail by the Doughty group, demonstrating a close correlation between the timing of tamoxifen administration and the cell lineage labeled, including CN GABA interneurons and different GABA interneurons of the cerebellar cortex, with a clear inside-out progression. This work showed that Purkinje cells express *Neurog1* around the time they become post-mitotic, while GIFM labeled both mitotic and post-mitotic interneurons (Obana et al. 2015).

Finally, a genome-wide analysis of gene expression was conducted in the cerebellar primordium of E11.5 *Neurog1* null mice to identify the *Neurog1* transcriptome in the developing cerebellum. This screen identified 117 genes differentially enriched in *Neurog1*−/− versus control sample sets with a high presence of gene sets enriched for functions in nervous system development. An in silico analysis of promoter regions identified high probability *Neurog1* regulatory (E-box) binding sites in many of the differentially expressed genes, sometimes accompanied by *Pax6* binding motifs in 25 of these 94 promoters, suggesting a Neurog1-Pax6 cross talk in the activation of some genes (Dalgard et al. 2011).

In summary, *Neurog1* is expressed in progenitors giving rise to PCs and GABAergic interneurons of the cerebellar cortex and nuclei. This gene appears to regulate a large number of downstream genes controlling development. A detailed analysis of *Neurog1* null cerebella is required to define the specific role of this gene in the ontogeny of the cerebellar territory.

Neurog2 Labels the PC Lineage and Regulates PC-Progenitor Cell-Cycle Progression and Dendritogenesis

Finally, a study by Florio et al. (2012) has addressed some of the roles played by *neurogenin 2* in cerebellar development. As mentioned, this *Atonal* homolog is expressed in the cerebellar VZ: at E12.5, its expression domain roughly coincides with those of *Ptf1a* and *Ascl1* (Zordan et al. 2008). Florio et al. replaced the only coding exon of *Neurog2*, namely, exon 2, with the *Cre-ER*^{T2} fusion gene (Imai et al. 2001). In this way, they generated a *Neurog2*-null knock-in allele expressing a tamoxifen-inducible Cre in a pattern faithfully recapitulating the *Neurog2* expression domain. Thus, they could use the expression of the human estrogen receptor as a proxy for *Neurog2* gene transcription. The gene is expressed at low levels in proliferating, neurogenic radial glia of the cerebellar VZ, in a pattern complementary to that of the Notch intracellular domain. By cumulative S-phase labeling with thymidine analogs, Florio et al. established for the first time that the duration of the cell cycle at E12.5, corresponding to the peak of Purkinje cell neurogenesis, is ~14 h. By calculating the duration of each cell cycle stage, they determined that, in the VZ, *Neurog2* is expressed mostly, albeit not exclusively, in G_1 progenitors. Embryos homozygous for the null allele progressed slowly through the cell cycle, stalling mainly in early G_1, leading to an overall reduction of the mature cerebellar volume. However, ectopic overexpression of *Neurog2* induced cell cycle withdrawal and precocious differentiation. Taken together, these results are compatible with a role for oscillating *Neurog2* protein levels in regulating and promoting cell cycle progression and with the notion that VZ cells upregulating *Neurog2* prior to the G_1 restriction point exit the cell cycle and start delaminating to undertake neuronal differentiation. By GIFM these authors showed that a large majority of *Neurog2* progenitors give rise to the PC lineage, with a minority of them contributing to the VZ-derived component of CN, mostly projection Smi32+ projection neurons, likely representing nucleo-olivary ones. Only in rare cases did cycling *Neurog2*+

progenitors give rise to Pax2+ interneurons or S100β+ astroglia. When transplanted heterochronically into the postnatal cerebellum, *Neurog2*+ cerebellar progenitors overwhelmingly gave rise to well-differentiated PCs.

In addition, these authors found that *Neurog2* plays an important role in the early stages of dendritogenesis, that is while this process is for the most part cell-autonomous, progressing independently of the influence of GCs. *Neurog2*-null PCs develop stunted and poorly branched dendrites since early stages of dendritogenesis. This important evidence was supported by the results of loss- and gain-of-function experiments alike.

Neurog2 is expressed by cycling progenitors cell autonomously fated to become PCs, even when transplanted heterochronically. During cerebellar development, *Neurog2* is expressed in G1 phase by VZ progenitors poised to exit the cell cycle. In the absence of *Neurog2*, both cell-cycle progression and neuronal output are significantly affected, leading to an overall reduction of the mature cerebellar volume. Although PC fate identity is correctly specified, the maturation of their dendritic arbor is severely affected in the absence of *Neurog2*. Thus, *Neurog2* is a regulator of PC development and maturation.

Conclusion

Proneural genes are expressed at crucial stages in cerebellar neurogenesis. Their ascertained roles include the regulation of neuronal fate determination, neuronal type specification, terminal differentiation, and GC clonal expansion. Under all those circumstances, they may be part of as yet unclarified combinatorial codes that integrate their function with that of many other genes, particularly those encoding other transcription factors. Further studies are required to dissect the molecular machinery in which they function, in cooperation with regulatory cascades controlling positional identity, fate specification, and differentiation.

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