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Cerebellar development and disease

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The molecular control of cell-type specification within the developing cerebellum as well as the genetic causes of the most common human developmental cerebellar disorders have long remained mysterious. Recent genetic lineage and loss-of-function data from mice have revealed unique and nonoverlapping anatomical origins for GABAergic neurons from ventricular zone precursors and glutamatergic cell from rhombic lip precursors, mirroring distinct origins for these neurotransmitter-specific cell types in the cerebral cortex. Mouse studies elucidating the role of Ptf1a as a cerebellar ventricular zone GABerigic fate switch were actually preceded by the recognition that *PTF1A* mutations in humans cause cerebellar agenesis, a birth defect of the human cerebellum. Indeed, several genes for congenital human cerebellar malformations have recently been identified, including genes causing Joubert syndrome, Dandy-Walker malformation, and pontocerebellar hypoplasia. These studies have pointed to surprisingly complex roles for transcriptional regulation, mitochondrial function, and neuronal cilia in patterning, homeostasis, and cell proliferation during cerebellar development. Together, mouse and human studies are synergistically advancing our understanding of the developmental mechanisms that generate the uniquely complex mature cerebellum.

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Introduction

The basic circuitry of the mature cerebellum has been known for more than 100 years; however, the developmental mechanisms that generate this complexity have only begun to be elucidated much more recently. The study of spontaneous and targeted mutations in mice that cause congenital ataxias has been fundamental to this progress. More recently, the use of powerful new genetic fate-mapping technology in cerebellar mutant mice has driven many of the new molecular insights of cerebellar development. Concurrently, multiple human cerebellar malformations have been delineated because of improvements in neuroimaging and improved classification of these disorders. This has fueled the identification of several disease genes leading to a new molecular classification of these disorders and permitted construction of mouse models to delineate the underlying pathogenesis. Together, mouse and human genetic approaches are synergistically driving significant progress toward an improved understanding of the basic mechanisms of cerebellar development.

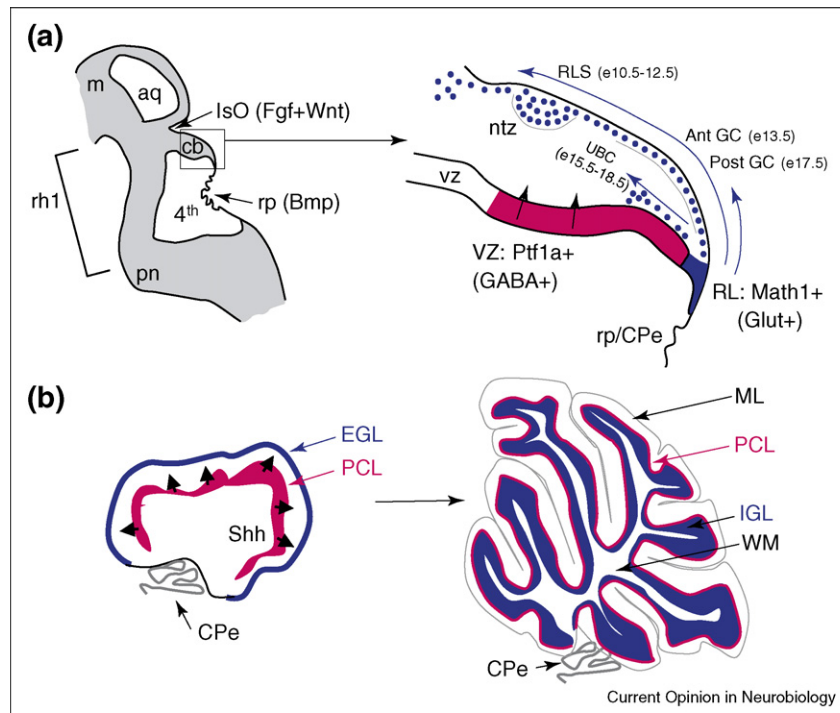
Overview of cerebellar development and recent findings in mice

Cerebellar morphogenesis

The cerebellum arises from dorsal rhombomere 1 of the anterior hindbrain and is positioned along the anterior/posterior axis of the neural tube by Fgf and Wnt signals from the isthmic organizer located at the midbrain–hindbrain junction [1]. The developing cerebellum is also influenced by the adjacent fourth ventricle roof plate, which secretes Bmp, Wnt, and retinoic acid [2••,3•] (Figure 1a). Mouse fate-mapping experiments have shown that during early embryogenesis, there is a 90° rotation of dorsal rhombomere 1 which converts the rostral–caudal axis of the early neural tube into the medial–lateral axis of the wing-like bilateral cerebellar primordia [4••]. As neurogenesis progresses, the bilateral primordia fuse on the dorsal midline over the fourth ventricle to establish the medial vermis and lateral cerebellar hemispheres [5].

Cerebellar neurons are generated from two anatomically and molecularly distinct progenitor zones within the primordia or cerebellar anlage. These are the cerebellar ventricular zone (VZ), expressing the bHLH factor Ptf1a, and the more dorsally located rhombic lip (RL), expressing the bHLH factor Math1 (Figure 1a). Newly differentiating neurons, including cerebellar Purkinje cells (PCs), are postmitotic as they leave the VZ and migrate radially within the developing anlage. By contrast, cells exiting the RL migrate over the anlage forming an external layer of cells that continue to proliferate. Granule neuron progenitors within this external granule layer (EGL) are driven to proliferate through the reception of a mitotic Shh signal received from the underlying differentiating PCs within the anlage (Figure 1b). Exten-

Figure 1



Overview of mouse cerebellar neurogenesis. **(a)** Schematic parasagittal section through the midbrain/hindbrain region of a mouse e12.5 neural tube. The cerebellum is a dorsal derivative of hindbrain rhombomere 1 (rh1) under the influence of signaling from both the isthmic organizer and fourth ventricle roof plate. Within the developing cerebellar anlage, distinct progenitor zones form marked by two distinct transcription factors, Math1 and Ptf1a. Math1 expression in the rhombic lip (rl) is induced by Bmp signaling from the roof plate (rp) which itself differentiates into the choroid plexus (CPe). Genetic fate-mapping studies have shown that Math1+ RL progenitor cells give rise to multiple glutamatergic+ derivatives in a time-dependent sequence. Early progenitors feed into the rostral migratory stream (RLS). The RLS migrates over the cerebellar anlage and gives rise to multiple brain stem precerebellar nuclei, including the pontine nuclei (pn). RLS cells next give rise to glutamatergic deep cerebellar nuclei (DCN), which settle into the nuclear transitory zone (ntz). Math1+ RL cells also generate cerebellar granule cells (GC) that form the cerebellar external granule layer in an anterior to posterior temporal gradient. Unipolar brush cells (UBCs) are the final Math1+ RL population and migrate through the cerebellar while matter. Concurrently, the ventricular zone (VZ) of the cerebellar anlage expresses Ptf1a. These progenitors exit the cell cycle, migrate radially into the cerebellar anlage and give rise to glutamatergic deep cerebellar nuclei (DCN), and cerebellar interneurons including Basket and Stellate cells. m: midbrain; aq: aqueduct. **(b)** Schematic midsagittal section of mouse cerebellum at day of birth. The Purkinje cell layer (PCL) is located underneath the EGL and secretes Shh which is received by EGL cells and drives their extensive proliferation such that granule cells become the most abundant neurons in the cerebellum and in the entire brain. Upon differentiation, EGL cells migrate through the PCL layer to form the IGL of the mature cerebellum. Their trailing axons form, the molecular layer (ML). Purkinje cells project into the cerebellar white matter (WM). Drawings are not to scale.

sive cell interactions and inward radial migration of EGL cells to form the IGL are required to achieve the final structure of the mature cerebellum [6,72•].

Distinct origins for GABAergic and glutamatergic cerebellar neurons

Recent loss-of-function and fate-mapping experiments have demonstrated that Ptf1a and Math1 are not only useful markers for each cerebellar progenitor zone but also essential for the generation of correctly specified progenitors within their respective germinal zones. In the absence of the Ptf1a, the cerebellar VZ fails to generate all of the known GABAergic cerebellar neuronal subtypes including PCs, stellate and basket cells and a subset of deep cerebellar neurons, which constitute the main outflow tract of the cerebellum [7•,8••]. Fate mapping of Ptf1a+ cells in wild-type mice demonstrates that Ptf1a+ VZ cells are normally fated to produce all of these GABAergic cerebellar cell types and thus, their loss is caused by a primary failure of the VZ to produce appropriate neurons in the absence of Ptf1a. Fate mapping in Ptfa1−/− mutants also demonstrates that some mutant cerebellar VZ progenitor cells aberrantly migrate and express typical RL markers such as Math1, Reelin, and Zic1/2, indicating a transformation of the mutant cerebellar VZ descendents to the adjacent RL cell fates. This suggests that the GABAergic fate choice in the cerebellum is based upon a relatively simple genetic switch [9].

It has long been known that the cerebellar RL expressing Math1 is the source of all cerebellar granule neurons [10,11]. Recent genetic fate mapping of Math1+ cells,

combined with newly developed cerebellar slice culture assays have completely revolutionized our understanding of the cerebellar RL. Surprisingly, the Math1+ RL gives rise not only to glutamatergic granule neurons but also to all known glutamatergic neurons of the cerebellum. These include both unipolar brush cells, which serve as a relay cell amplifying the excitatory effects of mossy afferent fibers on granule cells [12], as well as the glutamateric subset of deep cerebellar nuclei neurons [13–15,16••,17•,18] (Figure 1a). Thus, the well-ordered cellular organization of the mature cerebellum is achieved through the bipartite origins of its constituent neurons. Despite the complexity of the final mature structure, the cerebellum is not that different from the developing spinal cord and telencephalon, where distinctly ordered progenitors along the dorsal/ventral axis of the neural tube give rise to cells of distinct neurotransmitter phenotypes [18].

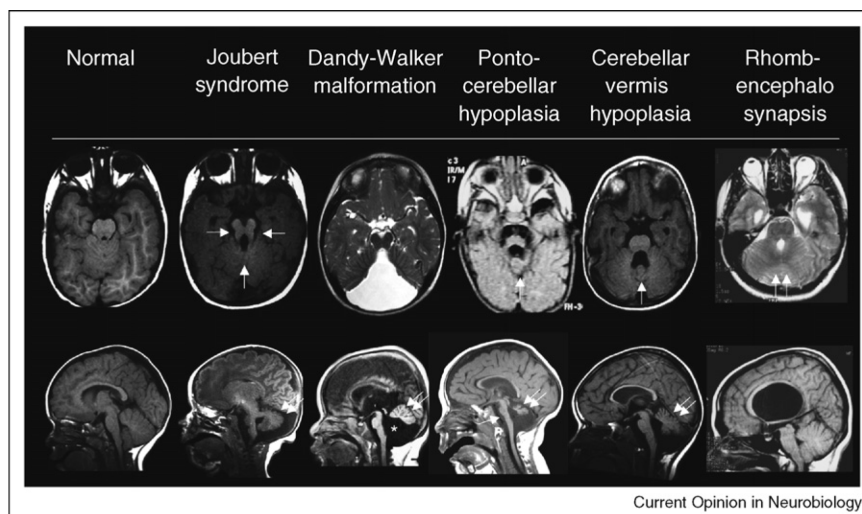
In contrast to the apparent switch in cell-fate Ptf1a−/− mutants, there is no evidence that Math1−/− cells transform their cell fate. In Math1−/− mutants, the RL still forms, but there is a failure to produce granule neurons. As a result, there is a failure to generate the EGL [19]. The new fate-mapping data reveal that all other Math1+ lineage cells also fail to be generated [13,14]. Thus, Math1 is not required for the formation of the RL or specification of neurotransmitter identity. On the basis of inducible fate mapping with Math1-CreER^{T2} mice, recent studies have even more surprisingly demonstrated that Math1+ cells are not the definitive stem cell population of the RL. Rather, Math1+ progenitor cells are continuously induced from an RL stem cell population that has yet to be identified [14].

So what are the mechanisms that segregate the VZ and RL? Recent studies have determined that both RL induction and Math1 expression is dependent on Bmp-derived signals from the adjacent roof plate [2••,20]. Further, the roof plate Bmp signal is countered by antagonistic Notch1 activity within the cerebellar VZ [21••]. Thus, antagonism between the Notch and BMP signaling pathways regulates the differentiation of cerebellar progenitors throughout the period of cerebellar neurogenesis.

Human congenital ataxias and their causes

The identification of *Ptf1a* as a major player in cerebellar neurogenesis has been a major recent advance in cerebellar developmental biology. Interestingly, *Ptf1a* (a gene well known for its role in pancreas development [22]) initially came to the attention of cerebellar biologists through human genetic analysis. Mutation in the *PTF1A* gene was first identified in a large family segregating both cerebellar agenesis and neonatal diabetes [23]. Subsequent work on this gene in mice revealed its crucial role in cerebellar GABAergic neuronal specification and also revealed that the complete cerebellar agenesis phenotype seen at birth in both humans and mice is actually a secondary phenotype. In the absence of *Ptf1a*, the failure

Figure 2



Spectrum of human cerebellar malformations on MRI. Top row: axial images at the level of the midbrain–hindbrain junction (isthmus). Bottom row: midline sagittal images. In normal, isthmus shape is relatively circular, and cerebellar vermis is full. In all of the conditions except rhombencephalosynapsis the cerebellar vermis is hypoplastic (double arrows on bottom row). In Joubert syndrome (JSRD) the isthmus takes the shape of a 'molar tooth' (arrows). In Dandy-Walker malformation (DWM), the posterior fossa is full of fluid (white field) and bottom shows the cystic dilatation of the fourth ventricle (*), which is rotated anteriorly. In pontocerebellar hypoplasia (PCH) the brainstem is also involved. Note small isthmus and vermis (arrow, top) and reduced brainstem volume (arrow, bottom). Cerebellar vermis hypoplasia (CVH) shows reduced vermis without other associated features. Rhombencephalosynapsis shows fusion of the two cerebellar hemispheres, and the vermis is replaced by this fusion (double arrows, top).

to generate GABAergic neurons secondarily leads to massive prenatal death of all cerebellar glutamatergic neurons because their GABAergic synaptic partners are not present [7•]. Together, the Ptf1a studies highlight the synergy between human and mouse genetics and suggest that human cerebellar congenital ataxias can greatly inform our understanding of the basic genes and mechanisms driving cerebellar development.

Overview of human congenital ataxias

Cerebellar agenesis is one of the many human congenital ataxias, which are a group of conditions that present in the first few years of life with motor disability, muscular hypotonia and incoordination, and impaired development. Characteristically, such patients display some degree of cerebellar malformation that typically involves the vermis, and which may accompany other developmental brain abnormalities and other non-CNS developmental abnormalities. Brain imaging studies have thus become a key element in distinguishing each malformation and has allowed for both clinical and genetic delineation of several distinct syndromes (Figure 2). Many of the newly identified causative genes have not previously been implicated in cerebellar development. The subsequent generation of related mouse models is just now beginning to illuminate many new aspects of cerebellar developmental biology.

Joubert syndrome and related disorders

Joubert syndrome and related disorders (JSRDs) are a group of recessively inherited conditions that are characterized clinically by congenital ataxia, hypotonia, episodic breathing dysregulation, and mental retardation [25]. The signature feature of this group of disorders is the 'molar tooth' sign. This sign is a specific malformation of the brainstem, cerebellum, and the cerebellar peduncles, which together, give the appearance of a tooth-like shape in axial MRI images at the level of the midbrain–hindbrain junction (isthmus) [26] (Figure 2). It has been recognized that many patients with Joubert syndrome identified by brain imaging studies also have retinal dystrophy and nephronophthisis (renal fibrocystic disease), two conditions related to defective function of primary cilia, cellular appendages of unclear function. Several recent proteomics and bioinformatics studies have identified lists of candidate cilia-functioning proteins [27]. These data, together with availability of large, consanguineous families segregating JSRD, have sped the discovery of several of the responsible JSRD genes. Currently, there are seven genetic loci that have been mapped for the various subtypes of Joubert syndrome, and the first five genes have been identified. These include *Ableson-helper integration-1* (*AHI1*), *Nephrocystin-1* (*NPHP1*), *Centrosomal protein-290* (*CEP290*), *Transmembrane protein 67* (*TMEM67*), and Retinitis pigmentosa GTPase regulator interacting protein-like (*RPGRIP1L*). Each of the genes encodes a modular scaffolding protein without clear enzymatic domains, but sharing several protein-interaction domains of unknown function, suggesting that they may be a part of a signaling complex [28••,29••,30,31,32••,33••,34•,35•,36•]

The Joubert syndrome connection with cilia

Although the function of JSRD proteins remains largely unknown, recent evidence suggests roles in either mediating the assembly/stability of cilia or mediating cargo transport within cilia. When tested directly, at least three of the encoded proteins, NPHP1, CEP290, and RPGRIP1L have demonstrated localization to the basal body or cilium [32••,35•,36•,37], further suggesting a role at the cilium or basal body. Remarkably, *CEP290* was concurrently identified as mutated in the *rd16* mouse [38••] and rdAc cat [39••] both models of retinal dystrophy. In these models there is a failure to transport rhodopsin to the photoreceptor outer segment and of disk morphogenesis. Because the photoreceptor outer segment is a giant modified cilium, the genetic evidence suggests a defect in ciliary function. Similarly, there is a failure to transport G proteins into the cilia of olfactory sensory neurons *rd16* mutant mice resulting in anosmia [40]. Finally, one JSRD patient with a proven *CEP290* mutation displayed complete *situs inversus*. Together, these data suggest a link between JSRD and other ciliopathies [41]. But what could be the role of cilia proteins in the developing cerebellum?

In the developing cerebellum, primary cilia have been identified ultrastructurally in both PCs and granule cell progenitors [42,43]. Indeed, primary cilia are appendages found on most eukaryotic cells, and are defined by a membrane-surrounded structure with a $9 + 0$ or $9 + 2$ microtubule structure and a basal body (centrosome) at the base. Primary cilia are differentiated from motile cilia found in many anatomic locations (Henson's node, respiratory epithelium, sperm cerebral ependyma), which display a patterned beating movement to produce fluid flow. The function of primary cilia is just now coming to the forefront of science, with the recent discovery of evolutionary conservation of many of the factors required for retrograde and anterograde transport in lower ciliated organisms.

The current hypothesis is that JSRD represent primary disorders of ciliary transport within cellular primary cilia in the developing cerebellum. This hypothesis is supported by the analysis of mice with conditional mutations in the *Kif3a* and *IFT88* genes, which encode intraflagellar transport proteins that are required for cilia formation and maintenance. These mice have cerebellar morphological defects that mirror those seen in JSRD. Mechanistically, conditional loss of *Kif3a* and *IFT* in the developing mouse cerebellum results in the failure of Shh-dependent proliferation of granule neuron progenitors within the developing cerebellar EGL [44•,45•] (Figure 1b). Because cilia are required to process both Shh and Wnt signals in a

range of cell types [46–49], it is hypothesized that the JSRD genes encode mediators of these signal transduction pathways at the primary cilium and that the primary defect in JSRD is compromised granule cell proliferation. This in turn, leads to significant cerebellar hypoplasia. Axonal migration defects causing the distinctive 'molar tooth' sign may also be caused by ciliary defects; however, until JSRD gene-specific knock-outs are available, these hypotheses cannot be directly tested.

Dandy-Walker malformation and cerebellar vermis hypoplasia

Dandy-Walker malformation (DWM) is the most common congenital malformation of the human cerebellum. DWM is characterized by a severe hypoplastic cerebellar vermis which is rotated away from the brainstem and a significantly enlarged fourth ventricle in an enlarged posterior skull [50,51]. Although there is cerebellar hypoplasia, DWM is not associated with the 'molar tooth' sign of JSRD and in contrast to JSRD, DWM very rarely segregates in a Mendelian fashion and has a very low recurrence risk in families [52•,53]. The identification of rare patients with chromosomal abnormalities has opened the door to DWM gene characterization.

The first DWM causative genes were identified by physical mapping of interstitial deletions of chromosome 3q24 (del3q24) in several DWM patients [54••]. This region encompasses the adjacent *ZIC1* and *ZIC4* genes, important members of the small *Zinc finger in cerebellum* family of transcription factors. Mice with heterozygous deletion of the orthologs of these two linked transcription factors have a phenotype that resembles human DWM. These mutants provide the first model to delineate the pathogenesis of DWM. Because Zic proteins interact with Gli proteins, which are obligate downstream components in Shh signal reception, it has been hypothesized that Zic proteins modulate Shh signaling [55–57] and may be involved in Shh-regulated EGL proliferation. The role of *Zic4* is not well understood, but Zic1 activity is required to maintain EGL cells in a progenitor state [57,58], though a direct role in the reception of Shh signaling has not been established. A diminishment in Shh-dependent granule neuron progenitor proliferation in *ZIC1/4*-dependent DWM may account for the cerebellar hypoplasia seen in these DWM patients; however, Zic functions that are Shh-independent may also contribute to the DWM phenotype [59,60], distinguishing DWM from JSRD. The study of *Zic* mouse models is ongoing, as is the search for additional human DWM causative genes [61].

The del3q24 DWM human phenotype is extremely variable ranging from classic DWM to mild cerebellar vermis hypoplasia (CVH) — a small cerebellar vermis that is not accompanied by the enlargement of the fourth ventricle or enlarged posterior skull that is seen in classical DWM [54••]. This suggests that CVH can represent one end of phenotypic spectrum with the same molecular pathogenesis as DWM. However, it is also clear CVH can also be distinct from DWM. For example, mutations in *Oligophrenin 1* (*OPHN1*), a widely expressed gene encoding a rhoGAP protein, cause X-linked mental retardation and CVH, but never DWM [62–64]. *In vitro* and *in vivo* experiments have demonstrated a role for *Ophn1* in dendritic spine morphogenesis in hippocampal neurons in mice, though no gross cerebellar anatomical abnormalities were observed in *Ophn1* mutant mice [65,66]. Thus, the basis of human *OPHN1*-dependent CVH remains obscure.

Developmental degenerative disorders

Numerous degenerative disorders of the mature cerebellum, known as spinal cerebellar ataxias (SCA), have been described in humans, many of which are caused by expanded CAG trinucleotide repeats leading to the progressive degeneration of PCs [67]. An intriguing recent paper demonstrates that compromising PC development, by expressing a toxic transgene early in development, contributes to the severity of the neurodegeneration in adult mice [68••]. These data suggest an overlap between development and degeneration, and that the eventual timing of the onset of degeneration is actually determined during prenatal life. Apparently, we can no longer think of development and degeneration as distinct entities. This is certainly the case with *PTF1A* mutations and cerebellar agenesis, as discussed above. A number of additional human cerebellar developmental degenerative disorders have been recognized and have been classified as pontocerebellar hypoplasia (PCH). PCH is characterized by the progressive atrophy of the ventral pons, inferior olive, and cerebellum, with onset during neonatal cerebellar development, but continuing after birth. At least three subtypes exist based on clinical and pathological features [50,69]. The first PCH gene has recently been reported as a loss-of-function of *RARS2*, encoding mitochondrial arginine-transfer RNA (tRNA) synthetase. This mutation was identified in a family with nonsyndromic PCH already evident at postnatal day 3 [70••]. Because *RARS2* is expressed in all cells, the mechanism of the neuron-specific phenotypes remains unclear. It will be fascinating to see this discovery translate into an improved understanding for the role of mitochondria in cerebellar development and homeostasis once appropriate mouse models are generated.

Conclusions

The cerebellum plays crucial roles in sensory integration, motor planning as well higher cognitive processing [71]. Despite its importance, we know surprisingly little about its development. The use of new fate-mapping strategies in mice has helped define unexpected origins for unique cellular populations within the cerebellum. Defining the genetic underpinnings of

some of the common causes of cerebellar malformations in humans holds the promise of improving diagnosis and prognostic information for these relatively common birth defects, as well as helping to uncover the unique molecular cues that are required for the development of the cerebellum across species.

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