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NEUROANATOMY

**Concise review on the developmental genetics of cerebellum and highlight bases of known cerebellar disorders**

**ABSTRACT**

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**ABSTRACT**

The cerebellum is one of the first brain structures to begin to differentiate, yet it is one of the last to achieve maturity — the cellular organization of the cerebellum continues to change for many months after birth. The internal structure of the cerebellum reflects an intriguing paradox; its cytoarchitecture is relatively simple and repeated throughout, yet the connections between its neurons are wired into a complex array of gene expression domains and functional circuits. The developmental mechanisms that coordinate the establishment of cerebellar structure and circuitry provide a powerful model for understanding how functional brain networks are formed. Two primary germinal zones generate the cells that make up the cerebellum. Each zone expresses a specific set of genes that establish the cell lineages within the cerebellar anlage. Then, cohorts of differentiated projection neurons and interneuron progenitors migrate into the developing cerebellum. Thereafter, a number of remarkable patterning events occur including transformation of the smooth cerebellar surface into an intricately patterned series of folds, formation of three distinct cellular layers, and the demarcation of parasagittal gene expression domains. Together, these structural and molecular organizations are thought to support the proper connectivity between incoming afferent projections and their target cells. After birth, genetic programs and neural activity repattern synaptic connections into topographic neural networks called modules, which are organized around a longitudinal zone plan and are defined by their molecular, anatomic, and functional properties.

**Overview of human cerebellar development**

The cerebellum develops from the dorsal region of the posterior neural tube. The embryonic cerebellum begins as little more than symmetric bulges into the early fourth ventricle: cerebellar hemispheres arise as mere buds from laminae on either side of the rhombencephalic midline, and the most rostral segment of the metencephalon produces outgrowths that form the first elements of the cerebellum. These lateral elements develop towards the midline and fuse in a rostral-to-caudal direction. As the primitive hemispheres come into contact with each other, they form first the superior and then the inferior vermis. The lateral elements from this fusion develop into the cerebellar hemispheres.

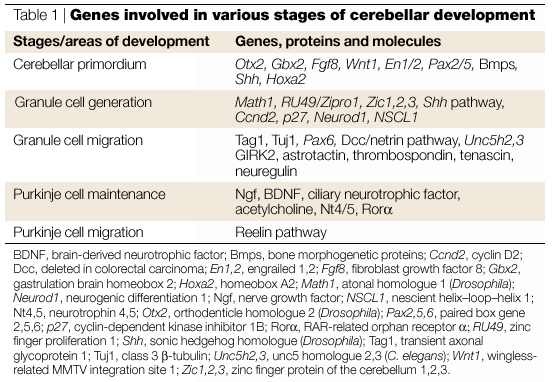
Cells in the cerebellum arise from two different germinal matrices. From the ventricular zone (also known as the ventricular germinal matrix), cells radiate laterally and evolve into the deep cerebellar nuclei and Purkinje cells of the cerebellar cortex. The first cells to be born become the deep cerebellar nuclei at about week eight in human embryogenesis. At week nine, the ventricular zone begins to produce cells that will eventually form the Purkinje neurons. By 24 weeks, these proto-Purkinje cells send dendrites to the PARALLEL FIBRES of the granule neurons. The full number of Purkinje cells is present early on, but their mature monolayer forms some time between 16 and 28 weeks postnatally. Purkinje cells continue their maturation after birth, projecting to the deep cerebellar nuclei and refining the input they receive from the CLIMBING FIBRES of inferior olivary neurons.

From the ventricular zone, a third population of neurons is born after the formation of Purkinje cells. These neurons include the stellate, basket and Golgi interneurons that can be found in the molecular layer. These three kinds of neurons have a modulatory action on the Purkinje cells and granule neurons.

Unlike most of the cell types of the cerebellum, which are born at the ventricular zone, cerebellar granule neurons come from a specialized germinal matrix called the rhombic lip. Migration of these primitive cells over the surface of the cerebellum starts as early as week 11 in humans; neuronal elements are present in the external granular layer (EGL) by week 27. From the EGL, a second zone of proliferation, the granule neuron precursors, migrate deeper into the cortex. This inward migration continues after birth, with the EGL disappearing within the first year of life in humans.

In the past decade, genetic studies of various mouse mutants became the primary source of information about cerebellar development

Table 1 Genes involved in various stages of cerebellar development



**Genes in the developing cerebellar primordium**

The neural tube can be thought of as comprising four different regions during early development. The most anterior portion of the neural tube, the prosencephalon, gives rise to the forebrain. The mesencephalon, just caudal to the prosencephalon, gives rise to the midbrain, whereas hindbrain regions evolve from the metencephalon and myelencephalon. CHICK–QUAIL CHIMAERA experiments have indicated that both the mesencephalon and metencephalon contribute to the developing cerebellum3,4,5.

The proper patterning of the mesencephalon and the metencephalon is dependent on molecular signals released from the ISTHMUS organizer (IO), which is located just caudal to the junction of these two regions Morphologically, this region is marked by a sharp bend of the neural tube. It has been shown in various mouse mutants, as well as in transplant experiments, that the IO is necessary and sufficient for patterning the mid-/hindbrain region from the neural tube. The IO is, in turn, set up by the expression of a complex array of genes. Two, in particular, are central to its development: Otx2 , one of the mouse homologues of the Drosophila gene orthodenticle , and Gbx2 , a homologue of the Drosophila gene unplugged 6. At embryonic day (E) 7.75, Otx2 is expressed in the mesencephalon, with a posterior boundary at the rostral metencephalon, whereas Gbx2 expression in the metencephalon is bounded anteriorly by the caudal mesencephalon7,8. The sharp boundary between the expression domains of these two genes reflects their reciprocal repression9,10 (For a thorough review of mid-/hindbrain patterning

In addition to helping form the IO molecularly, Gbx2 and Otx2 also regulate the expression of Fgf8 (fibroblast growth factor 8); Otx2 negatively regulates Fgf8 expression, whereas Gbx2 maintains it9,10,14. Fgf8 is involved in regulating the various genes expressed in the mid- and hindbrain regions. Mutant mice with a reduced level of Fgf8 expression have a severe patterning defect of the mid-/hindbrain region, which usually affects the cerebellum15.

Fgf8 is a diffusable factor that exerts its action partially by inducing the expression of wingless homologue 1 ( Wnt1 ) through Lim homeobox 1b ( Lmx1b )16,17. Wnt1, in turn, maintains the expression of Engrailed ( En1 )18, which then positively regulates Fgf8 expression, completing the feedback regulatory loop6. Mutants of Wnt1, En1 and Lmx1b all show patterning defects in the mid-/hindbrain region19,20 (R. Johnson, personal communication). Wnt1 and Lmx1b probably exert their influence through the action of En1 (Ref. 18). En2 , a paralogue of En1, might also function in mid-/hindbrain patterning. En2 is expressed shortly after En1. Deletion of En2 against a haploinsufficient En1 mutant background was accompanied by a patterning defect in the mid-/hindbrain region more severe than that seen in a single mutant of En1; similarly, deletion of En1 against a haploinsufficient En2 mutant background also leads to an exaggerated phenotype21.

Although the cross-regulation between Wnt1, En1 and Fgf8 is beginning to be understood, several other genes that are not part of this pathway are also

important in patterning of the mid-/hindbrain region. The paired box genes Pax2 and Pax5 are expressed in the mid-/hindbrain region21. Pax2-null mice never develop a cerebellum or posterior mesencephalon22. Although Pax5 mutants have only a mild phenotype in the mid-/hindbrain region, mice with a Pax5 mutation against a Pax2-sensitized background lack a cerebellum and posterior midbrain23. Pax2 and Pax5 might also be involved in the regulation of En1, Wnt1 and other patterning genes, and together constitute another positive regulatory loop9.

The Hox gene family, which has an active role in patterning the hindbrain, seems to help to restrict the development of metencephalon structures into the myelencephalon. For example, Hoxa2 (homeobox A2), the most anteriorly expressed Hox gene, probably marks the caudal limit of the cerebellar anlage at rhombomere 1 (Ref. 24). Mice without Hoxa2 develop enlarged cerebella24.

Less is known about the dorsoventral patterning in this region. Bone morphogenetic proteins (Bmps) and sonic hedgehog ( Shh ) govern neuronal fates in the spinal cord; they have also been implicated in dorsoventral patterning of the mid-/hindbrain region. Bmps can induce the cerebellar granule neuron marker mouse atonal homologue 1 ( Math1 ) when expressed in the ventral neural tube of the region25, and ectopic expression of SHH in the chick dorsal neural tube leads to ventralization of the neural tube and disruption of the mid-/hindbrain region26. Cerebellar development is also affected by ectopic expression of Shh, which leads to defects of the midline of the neural tube26.

In sum, the reciprocal repression of Otx2 and Gbx2 forms the IO, which in turn uses Fgf8 and En1 to pattern the prospective mid-/hindbrain region. Cells from both the mesencephalon and the metencephalon give rise to cerebellar tissues.

**Development of Purkinje cells**

The Purkinje, Golgi, stellate and basket cells all arise from the ventricular neuroepithelium3,5. Purkinje cells are born around E13, at which time they exit the cell cycle and migrate along the radial glial fibre system into the cerebellar anlage3.

Relatively little is known about the specific factors that govern Purkinje cell differentiation. Shortly after their final mitosis at E14, Purkinje cells begin to express the calcium-binding protein calbindin3. Calbindin-positive cells migrate from E14–E17 in a radial direction over the already formed deep cerebellar nuclei3. These Purkinje cells then settle and become suspended beneath the EGL, awaiting the inward migration of granule neurons27. The timely arrest of migration is dependent on the reelin pathway27,28. Mutations in the Reln gene or in components of its signalling pathway lead to various cerebellar defects (reviewed in Ref. 29). Although Purkinje cells depend on signals from the granule neuron precursors to migrate, their differentiation programme seems to be independent of granule neurons. Mutants that lack granule neuron precursors, such as weaver or Math1-null mice, seem to have differentiating Purkinje cells at this stage30,31.

In late embryogenesis, climbing fibres from the inferior olivary nucleus start to innervate Purkinje cells3. Extensive interactions occur between the climbing fibres and the Purkinje cells, and these interactions are believed to influence Purkinje cell development3. Different markers, such as NST-1 (Hsp70-4, heat-shock protein), are expressed at the time of contact3. At the same time, Purkinje cells are eliminating supernumerary climbing fibre synapses in several phases, at least one of which (during postnatal days 15–16) is activity- and NMDA (N-methyl-D-aspartate)-receptor-dependent32.

During their final maturation phase, Purkinje cells develop extensive dendritic arbors and synapse onto granule neurons3,33. As might be expected, this phase of development depends on granule neuron signals30,34. In mutants such as weaver, which do not have granule neurons, dendritic trees of Purkinje cells are altered30. Furthermore, culturing of Purkinje cells in vitro requires co-culturing with granule neurons for proper dendritic arborization34. Given the known role of some of the Wnt genes in axon and dendrite development, Wnt3 is a good candidate for influencing this phase of development. Wnt3 is expressed in Purkinje cells during this period, and its expression is dependent on granule neurons35.

Throughout the course of development, various growth factors are important for Purkinje cell survival33. Nerve growth factor, acetylcholine, neurotrophin 4/5, brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor have all been shown to have a positive effect on Purkinje cell number in vitro33,36,37. Similarly, the Rorα (RAR-related orphan receptor α) gene, which is mutated in staggerer mice, is also important for the survival of Purkinje cells38. In staggerer mice, although Purkinje cell genesis is normal, these neurons degenerate after E17 (Ref. 38). Apoptosis also controls Purkinje cell number; for example, transgenic mice overexpressing Bcl2 (B-cell leukaemia/lymphoma 2), a protector against apoptosis, have more Purkinje cells than do wild-type mice39.

**Development of rhombic lip and granule neurons**

Unlike all other cerebellar cells, which derive from the ventricular zone, the granule neurons derive from a separate germinal epithelium known as the rhombic lip (Fig. 3). The rhombic lip is located between the fourth ventricle and the ROOF PLATE in the metencephalon40. Along the anteroposterior axis, the rhombic lip extends from the first rhombomere to the eighth40. A source of dividing progenitors, the rhombic lip might also be able to induce other cells to adopt its fate through some diffusable signal40,41. For example, cells from the ventral neural tube can adopt the fate of granule neurons when they are interspersed with rhombic lip cells

Expression of the Math1 gene governs the germinal epithelium of the rhombic lip31. Math1 is expressed in the mid-/hindbrain region as early as E9.5, and persists in the rhombic lip and many of its derivatives42,43. Math1-null mice lack several rhombic lip derivatives, including the granule neurons of the cerebellum and the pontine nucleus of the precerebellar system31,43. The defect can be traced back to as early as E10.5, with a marked reduction in the proliferating cell population in the rhombic lip region31.

Chick–quail chimaera experiments have shown that the rhombomere 1 region of the rhombic lip is the probable source of granule neuron precursors40. This is further supported by the analysis of Hoxa2 mutants, which have an expanded rhombomere 1 and concomitant increase in granule neurons24. Inside the rhombic lip, granule neuron precursors proliferate and then assume a unipolar morphology, with a single process that projects away from the rhombic lip. They begin to migrate out from the rhombic lip at E13, and spread over the roof of the cerebellar anlage to populate the EGL40,41,44 (Fig. 3). At this point, the granule neuron precursors are still expressing Math1 and nestin, which labels undifferentiated precursors, but they also begin to express several other markers, including RU49/Zipro1 (zinc finger proliferation 1), Zic1 and Zic3 (zinc finger proteins of the cerebellum) (Refs 42,44,45). These precursors will become granule neurons when placed into the postnatal EGL44. This indicates that, fairly early on in development, rhombic lip cells are specified to become granule neurons, and are competent to respond to proper signals for differentiating into granule neurons. It is unclear, however, exactly what the roles of these transcription factors (Math1, RU49/Zipro1, Zic1 and Zic3) are during the migration of rhombic lip cells.

Rhombic lip cells continue to migrate to the cerebellar anlage and settle on its periphery to become the EGL (Fig. 3). The outer EGL is another zone of proliferation3. Granule neuron precursors in the outer EGL express several markers, including Math1, RU49/Zipro1 and Zic1. Although the role of Math1 at this outer EGL stage of development is not known, both RU49/Zipro1 and Zic1 are thought to participate in cell proliferation. Although RU49/Zipro1-null mice do not have obvious defects of cerebellar granule neurons, the overexpression of RU49/Zipro1 leads to an increase in the number of granule neurons and an increase in proliferation in the outer EGL46. Zic1-null mice have smaller cerebella and FOLIATION defects47, probably because of a reduction in the number of proliferating cells in the outer EGL, as shown by 5-bromo-2-deoxyuridine labelling47. Mutations of ZIC2 and ZIC3, which are also expressed in the EGL, are associated with neural tube defects, and ZIC3 mutations are associated with SITUS INVERSUS48,49. At least one human patient with the ZIC3 mutation is known to have cerebellar hypoplasia (B. Casey, personal communication); further studies in animal models will give us a better idea about the roles of Zic2 and Zic3 in the development of the cerebellum.

Cell proliferation at this stage seems to require interaction with Purkinje cells in addition to the milieu of the EGL33,50. Purkinje cells, in particular, are important in controlling cell proliferation of the outer EGL precursors33. Ablation of or defects in Purkinje cells lead to a decreased number of granule neurons33. It is believed that Purkinje cells release a diffusable factor such as Shh to control proliferation of the granule neuron precursors51,52. In vitro culture experiments show that Shh can stimulate proliferation significantly51,52. Mutations in patched, which activates the Shh pathway, have been linked to BASAL CELL NAEVUS SYNDROME (BCNS)53. Medulloblastoma, a tumour thought to be of granule cell precursor origin, occurs in some patients with BCNS53. These data strongly indicate that the Shh pathway is involved in the control of granule neuron proliferation by Purkinje cells. Interestingly, Zic proteins have been shown to modulate the activity of the Gli (GLI–Kruppel family) proteins, which in turn mediate Shh function54. So, Zic1 might control cell proliferation by indirectly affecting the Shh pathway.

Genes involved in the cell cycle are also important in cerebellar granule neuron proliferation. Cyclin D2 is a brain-specific cyclin that is found in the cerebellum55. Animals that lack cyclin D2 have fewer granule neurons and stellate interneurons55. The defect in the granule neuron population results from both a decreased number of proliferating precursors and an increase in cell death55. This indicates that some of these neuron-specific cyclins are involved in regulating the cell cycle in the cerebellum.

From the outer EGL, granule neuron precursors migrate into the inner EGL3. Cells in the inner EGL are postmitotic and are in a state of premigration56. The change from proliferating to postmitotic states might involve the accumulation of cell-cycle inhibitors. One such inhibitor, p27/Kip1 (cyclin-dependent kinase inhibitor 1B), is expressed in the inner two-thirds of the EGL, partly overlapping with the proliferating pool in the middle third of this region57. Mice that lack p27/Kip1 have an increased level of proliferation in the EGL and so a larger cerebellum57.

In the inner EGL, granule neuron precursors no longer express Math1; instead, they express two other basic helix–loop–helix transcription factors, Neurod1 and NSCL1 (Refs 58,59). Neurod1 is important for the survival of inner EGL granule neuron precursors, as Neurod1-mutant mice have an increased level of apoptosis58. Misexpression of NSCL1 in mitotic cells reduces proliferation, which indicates that NSCL1 might be involved in cell-cycle regulation60. Several trophic factors, including BDNF and insulin-like growth factor 1 (IGF1), have been shown to have a modulatory effect on apoptosis, which, of course, is an important mechanism for controlling cell number during development33.

The next stage of development for granule neuron precursors is the inward migration into the inner granule layer (IGL). At this point, the cells are expressing mature markers such as Tag1 (tubulin-associated glycoprotein 1) and class 3 β-tubulin/Tuj1 (Refs 27,61), and develop the parallel fibre, an axon extending from the cell body27. In addition, these cells now express Dcc (deleted in colorectal carcinoma), Unc5h2 and Unc5h3 , all components of the Dcc/netrin pathway known to be involved in axon projection and neuronal migration40. Several genes have been proposed to have a role in setting the stage for migration. Pax6 (paired box homeotic gene 6) is expressed in both the outer and inner EGL56. In Pax6/small eye (Sey)-mutant mice, the cerebellum is abnormal and lacks fissures56. There is no organized premigratory zone in the mutants, and the postmitotic granule neuron precursors are scattered in the EGL56. Although the granule neuron precursors in Sey mice express Tag1, there is no evidence of Unc5h3 expression56. In explant culture, neurite formation is also disturbed in sey mutants56. It was recently found that a DOMINANT-NEGATIVE form of either Unc51.1 or Unc51.2, both serine/threonine kinases, inhibits neurite formation when introduced into granule neuron precursors61.

Other factors are probably involved in preparing the granule neuron precursors for migration. At this stage, these precursors are experiencing profound electrophysiological changes33. Mutations in an inward-rectifying K+ channel, GIRK2 (weaver), cause these neurons to die33. It is thought that NMDA receptor activation is involved in preparing for migratory behaviour, and that the GIRK2 channel is responsible for compensatory hyperpolarization after NMDA-receptor-induced depolarization33. Mutations in GIRK2 therefore lead to sustained depolarization and death33.

From the inner EGL, granule neuron precursors migrate into the IGL under the guidance of radial glial fibres. Neurons interact with glia through a specialized process called the interstitial junction. This junction marks a widening of the intercellular space, with filamentous material in this space spanning the cleft and membranes to connect to the cytoskeletal elements27. Several proteins are known to be important in this migration. Astrotactin, an extracellular protein with epidermal growth factor (EGF) repeats and fibronectin repeats27, serves as a heterophilic ligand for glial binding62. Disruption of granule neuron migration has been seen after application of antibody against astrostatin and in astrotactin-deficient mice27. Thrombospondin and tenascin are two extracellular proteins involved in axon extension in the migrating neuron. Thrombospondins are extracellular glycoproteins that also bind calcium63. Disruption of thrombospondins by the application of antibody in vitro disrupts granule neuron migration27. Tenascin, containing fibronectin repeats and EGF repeats, is thought to promote neurite outgrowth and granule neuron migration64. Neuregulin, which contains an EGF-like domain and an immunoglobulin-like domain, is expressed in granule neurons and binds to ErbB4 (avian erythroblastosis oncogene 4) on the glial cell during migration27. Disruption of this interaction leads to abnormal migration27.

The final stage of maturation of granule neurons occurs in the IGL. At this point, granule neurons are expressing mature markers such as GC5 and GABA receptors65. MOSSY FIBRES from the precerebellar nuclei contact the granule neurons. This developmental process is partly controlled by Wnt7a , which is released by granule neurons66. At the same time, granule neurons extend connections to Golgi cells, a process controlled by contactin

COMPARTMENTILIZATON OF CEREBELLUM

The only gene that has been studied specifically for its role in compartmentalization is En2. This gene starts to be expressed slightly after its paralogue En1. En2 mutants show altered expression of En1, Pax2 and Wnt7b, and a delay in midline fusion as well as foliation defects71,73. En2 also negatively regulates L7/Pcp2 (Purkinje cell protein 2) expression, which occurs in a band adjacent to that of En2 expression. Ectopic expression of En2 under the L7/Pcp2 promoter leads to Purkinje cell death and foliation abnormalities, as well as sagittal banding of several markers (zebrin I, zebrin II and NADPH diaphorase

Human Cerebellar Malformations

cerebellar vermis hypoplasia (CVH), DWM, Joubert syndrome and related disorders (JSRD), and pontocerebellar hypoplasia (PCH)An external file that holds a picture, illustration, etc.
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The defining features of these diagnoses are based on imaging criteria rather than clinical outcome, with most of these diagnoses associated with intellectual and motor disabilities. CVH is characterized by a small hypoplastic cerebellum with the vermis more affected than the hemispheres. DWM includes CVH; however, there is also an upward rotation of the cerebellar vermis that results in an enlarged fourth ventricle, and an increased size of the posterior fossa. DWM is the most common cerebellar malformation, with an estimated incidence of approximately 1 in 5,000 CVH is also relatively common and often confused with DWM, making estimations of incidence problematic. CVH and DWM often present as sporadic cases, although there are several CVH loci with known recessive or X-linked inheritance Mendelian inheritance for DWM is rare, and the genetics are likely oligogenic In contrast, JSRD are most often autosomal recessive disorders and are rare, with a population incidence estimated to be 1/100,000 .As a group, JSRD are characterized by cerebellar vermis hypoplasia plus the presence of elongated cerebellar peduncles and a deepened interpeduncular fissure that appear as a “molar tooth” on axial brain scans. In addition, these patients exhibit axon guidance defects that include a decussation failure of the pyramidal tract and superior cerebellar peduncles. Patients with PCH exhibit a heterogeneous set of malformations characterized by hypoplasia and atrophy of the cerebellum, inferior olive, and ventral pons. This degenerative disorder often begins with embryonic atrophy of these regions.

Causative Genes in Human Cerebellar Malformations

In the last decade, there has been considerable effort in defining the genetic basis of human cerebellar malformations. Causative genes include those involved in cerebellar patterning, cell fate specification, and other developmental processes (Table 1).

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| Cerebellar malformations | Implicated human genes | Likely disrupted process |
| Cerebellar vermis hypoplasia (CVH) | OPHN1 [59, 60] | Spine morphogenesis |
| Dandy–Walker malformation (DWM) | ZIC1, ZIC4 [65], FOXC1 [17] | Granule cell differentiation |
| Joubert syndrome and related disorders (JSRD) | AHI1 [67, 68], ARL13B [69], CCD2A [70, 71], CEP290 [72, 73], INPP5E [74, 75], NPHP1 [76, 77], RPGRIP1L [78, 79], and TMEM67 [80] | Granule cell proliferation |

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| --- | --- | --- |
| Pontocerebellar hypoplasia (PCH) | CASK [86], RARS2 [88], TSEN54, TSEN34, and TSEN2 [89] | Spine development, cell proliferation, tRNA splicing, cellular maintenance. |

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