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Hassan Marzban *Editor*

Development of the Cerebellum from Molecular Aspects to Diseases

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Development of the Cerebellum from Molecular Aspects to Diseases

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Preface

For the past centuries, scientists have worked hard to understand the development of the cerebellum and its disorders. Cerebellar neurodevelopmental disorders are impairments of the growth and development of the cerebellum that result in birth defects that may have a severe impact on patients' lives. A narrower use of the term refers to a disorder that unfolds during individual development to affect motor function, emotion, learning ability, self-control, and memory. Knowledge obtained from advanced neuroimaging techniques and from molecular and genetic studies has provided rapidly growing evidence that the cerebellum is a brain region that is highly impacted by developmental defects.

Advanced technologies for research have changed our views of cerebellar development. This has led us to identify many of the biological, genetic, and environmental factors that contribute to the development and progression of cerebellar disorders. We believe that basic knowledge of the cerebellum, one of the most complex structures in the brain, will empower cerebellar researchers, neuroscientists, neurologists, and neurosurgeons every day.

This book covers diverse aspects of cerebellar development from different points of view including the epidemiology of cerebellar genetic disorders, developmental anatomy, cell biology, genetics, epigenetics, infectious diseases, and mechanisms involved in regulation of cell fate, while focusing on information that is relevant to clinicians serving patients with cerebellar disorders. This book consists of chapters written by experts in the field of cerebellar development, and it covers diseases related to the cerebellum, along with their epidemiology, clinical features, assessment, and management. To the extent that the book is a useful reference for neuroscientists and clinicians, we will have succeeded.

Winnipeg, MB, Canada — Hassan Marzban, PhD

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The Development of the Cerebellum: From the Beginnings

Jan Voogd

Abstract Sotelo stated in his introduction for a consensus paper on cerebellar development (Leto et al., Cerebellum 15:789–828, 2015) that "The work done in the late nineteenth century until the late 1970s provided substantial and significant information; however, it was only descriptive and barely addressed the mechanisms involved." Observations and their description, the nomenclature that evolved from these studies, and the ideas they fostered, indeed, formed the basis for our understanding of the mechanisms that underlie the complex development of the cerebellum, to be reviewed in this volume. This chapter will highlight some of these early contributions to the origin of the cerebellum, its histogenesis, the migration of its neurons, the development of the longitudinal Purkinje cell zones, their target nuclei and their connections, and the folial pattern of the cerebellum.

The Origin of the Cerebellum

The study of cerebellar embryology begins with His' [52, 53] description of his Rautenlippe (rhombic lip) in a human embryo. In the fifth week the "dorsal rim (of the rhombencephalic alar plate) curves laterally and forms a fold which surrounds the entire rhombic cavity …" (Fig. 1). His divided the rhombencephalon and its rhombic lip, into rostral and caudal portions. The rostral (upper) rhombic lip will give rise to the cerebellum, the caudal (lower) rhombic lip to several precerebellar nuclei. The upper rhombic lip develops in two, bilateral swellings connected by a thin midline portion (Fig. 2). At the midline, the cerebellum increases in bulk by the development of the cerebellar commissures and possibly by fusion of the intraventricular bulges. More recently the term "upper rhombic lip" is restricted to the posterior rim or germinal trigone of the cerebellar plate, with its attachment of the epithelial roof of the fourth ventricle, that should be distinguished from the ventricular zone, the neuroepithelium that covers its ventricular surface (Fig. 3).

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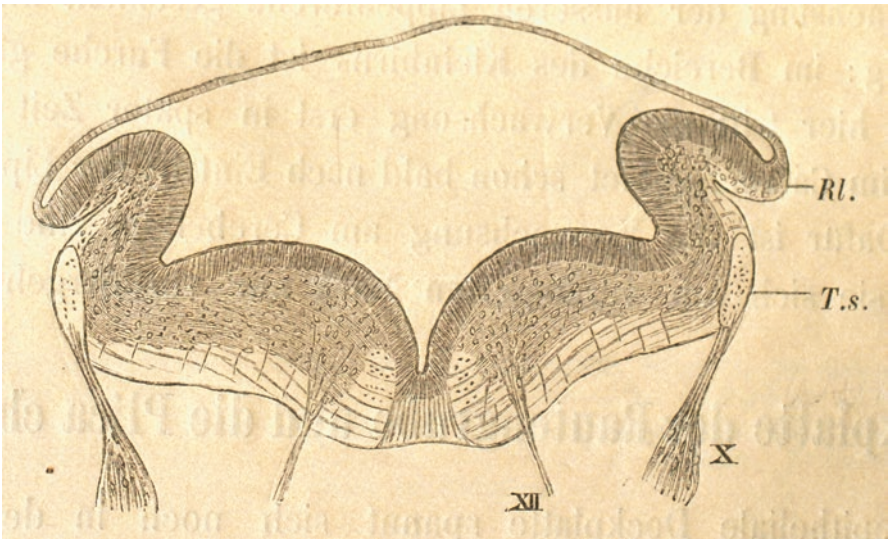

Fig. 1 The rhombic lip of a human embryo at the level of the greatest width of the fourth ventricle (Reproduced from His [52]). *Abbreviations*: *R.l* rhombic lip, *Ts* solitary tract, *X*, *XII* vagal and hypoglossal nerves

The general opinion was that the cerebellum originates from the dorsal portion of the first rhombomere [100, 111]. Several papers used the quail-chick marker system to trace the origin of the cerebellum. Substitution of the mesencephalic vesicle in chickens with a quail transplant resulted in the replacement of Purkinje cells and ependyma in the rostral cerebellum by cells with the typical massed quail chromatin in their nucleoli. According to Martinez and Alvarado-Mallart [80], these cells are present in a broad medial stripe, where labeled and non-labeled Purkinje cells occur together. Labeling is also found of the granule cells. According to Hallonet et al. [45], the labeled Purkinje cells are found in a V-shaped, rostral region reaching caudally to lobule VIII (Fig. 4). These authors denied the labeling of granule cells and concluded that these cells originate exclusively from the metencephalon, confirming the general opinion on this matter. Martinez and Alvarado-Mallart suggested that the rostral cerebellum might originate from the isthmic rhombomere, whereas its middle portion is a derivative of the first rhombomere. Its caudal portion, including the auricle and part of the avian lateral cerebellar nucleus, is derived from the second rhombomere [79]. Sgaier et al. [104] and Nieuwenhuys and Puelles [87] pointed out that the cerebellar anlage rotates from an original rostrocaudal, to a medio-lateral position, due to the development of the pontine flexure (Fig. 2). Purkinje cells produced by the ventricular zone maintain their medio-lateral position in the adult cerebellum. Those produced by the most medial (presumably isthmic rhombomere-derived) ventricular zone become located in the future vermis, subsequently more lateral parts of the ventricular zone give rise to Purkinje cells of more lateral parts of the hemisphere [4]. Granule cells produced by the upper rhombic lip do not maintain their original medio-lateral position in the adult, due to their latero-medial tangential migration in the external granular layer (EGL).

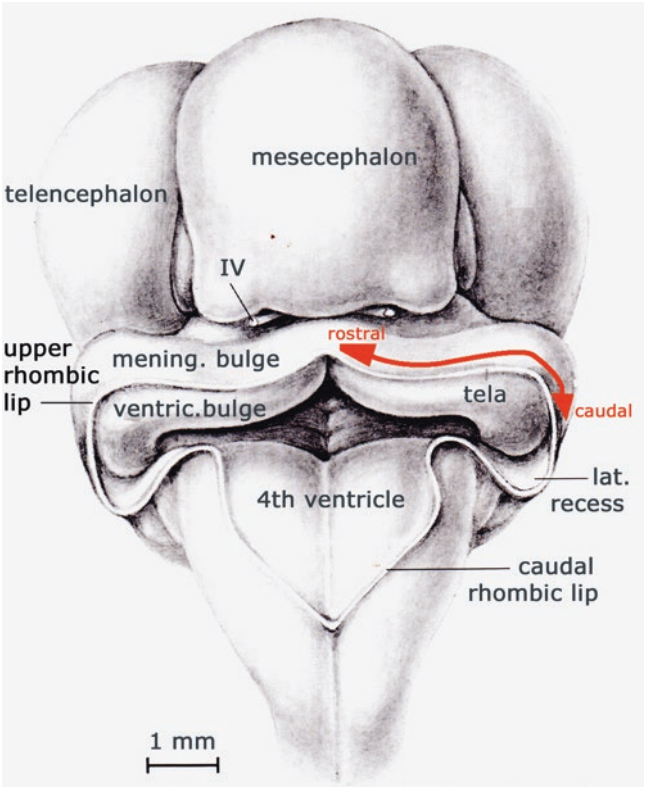

Fig. 2 Cerebellum of a 15-week human embryo (Reproduced from Nieuwenhuys et al. [88]). *Red arrow* indicates position of rostrocaudal axis of the cerebellar anlage after the rotation of the cerebellar anlage due to the pontine flexure

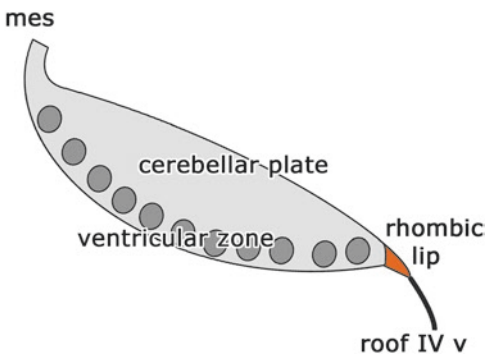

Fig. 3 Diagram of a sagittal section, showing the division of the germinal zone of the early cerebellar plate into the ventricular zone that will give rise to inhibitory neurons and the upper rhombic lip that produces the excitatory neurons of the cerebellum (Redrawn from Goldowitz and Hamre [40])

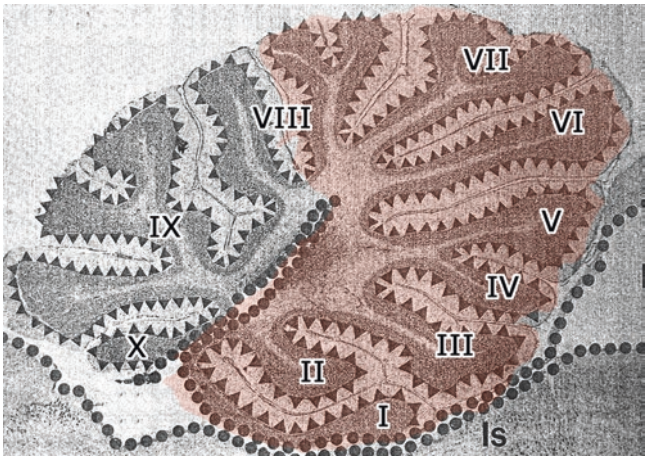

Fig. 4 Sagittal section through a chicken-chimera cerebellum. In the rostral region of the cerebellum, Purkinje cells (*triangles*) and ependyma (*circles*) are replaced by quail cells derived from the mesencephalon (Modified from Martinez and Alvarado-Mallart [80])

Histogenesis

The ventricular zone and the rhombic lip produce different types of neurons in successive waves. According to the autoradiographic studies of Miale and Sidman [81] and Pierce [93] in mice, using the incorporation of radioactive thymidine at their last mitosis, the large (glutamatergic) neurons of the cerebellar nuclei are born early at E10 and E11 in the ventricular zone and medium and small (presumably inhibitory inter- and nucleo-olivary neurons) between E11 and E17. Purkinje cells are born during the same period (E11–E13). Golgi cells in mice are produced by the ventricular zone in the 12- to 15-day embryo. After E15, dividing cells are present in the white matter throughout the cerebellum [81]. These cells give rise to Bergmann glia, astrocytes, oligodendrocytes, and basket and stellate cells of the molecular layer [126, 127]. In the rat, unipolar brush cells are born in the ventricular zone after the cessation of the production of the Purkinje cells. Lugaro cells develop in the same period as the Golgi cells [103]. Cells of the EGL arise from the caudal border of the cerebellar anlage (the upper rhombic lip) after E13. The EGL produces granule cells till well after birth (for similar data on the rat, see Altman and Bayer [5], for the monkey Gould and Rakic [42], for the chick embryo Kanemitsu and Kobayashi [57]). The more recent conceptual revisions of the origin of the neurons from the ventricular zone and the rhombic lip include the origin of the large glutamatergic neurons of the cerebellar nuclei from the rhombic lip and their inhibitory neurons from the ventricular zone (reviewed by Wingate in Leto et al. [74]) and the observation of Englund et al. [35] that unipolar brush cells are produced by the rhombic lip. Inhibitory neurons of the cerebellum, therefore, are produced by the

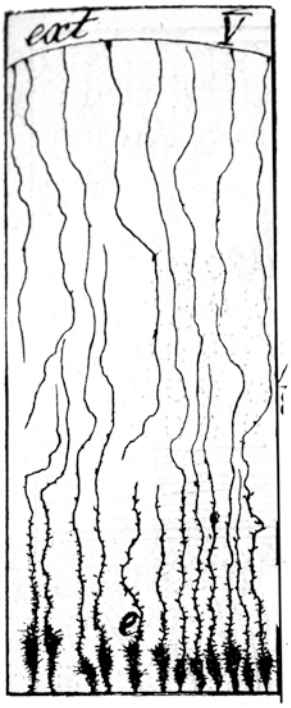

Fig. 5 Neuroepithelial cells (Popoff's spongioblasts) in a 1–1/4 cm cat embryo. Golgi method (Reproduced from Popoff [95]). *Abbreviations*: *e* ventricular zone, *ext* external limiting membrane

ventricular zone, excitatory neurons by the rhombic lip. Glutamatergic nuclear neurons and cells of the EGL are produced sequentially by the rhombic lip.

In their migration to the meningeal surface of the cerebellum Purkinje cells are supposed to use the processes of the neuroepithelial cells whose conical endfeet form the external limiting membrane (Fig. 5). A map of these processes that would predict the paths of migrating Purkinje cells is not available. In mice, migrating Purkinje cells at E15 avoid the cerebellar nuclei; at E17 they pass across them [125]. In the rat, all Purkinje cells migrate through the more superficially located transitory nuclear layer [3]. The clustering of the migrated Purkinje cells that will lead to the development of longitudinal Purkinje cell zones will be considered in another paragraph.

In his Golgi studies, Cajal [22, 23, 25] and his followers [10, 76, 95] distinguished different phases in the development of the Purkinje cells (Fig. 6a–d). In the first phase of the "disoriented dendrites," multiple processes arise from all over the cell body. The axon, first devoid of collaterals, enters the white matter. In the next stage, oriented and regular dendrites arise as a flattened tree from the upper pole of the cell. The axon emits multiple collaterals. Finally the processes of the cell body are resorbed, the dendritic tree acquires its definite shape, and many of the axonal collaterals are resorbed. Differentiation of the Purkinje cells is more advanced in the apices of the lobule. Purkinje cells of the rat mature early in lobules I and II and proximal V and VI and IX and X and late in distal VI, VII, and VIII [2, 41].

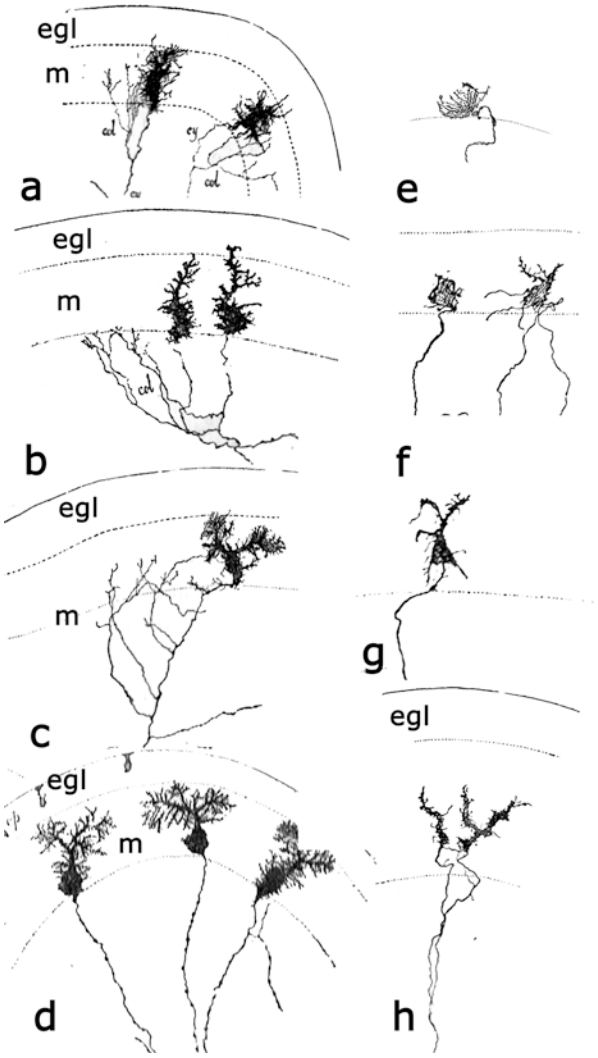

Fig. 6 Stages in the development of Purkinje cells (**a**–**d**) and climbing fibers (**e**–**h**) (Reproduced from Athias [10])

The development of the climbing fibers closely follows that of the Purkinje cell [22, 25, 95]. Cajal distinguished an early pericellular nest stage where the climbing fiber forms an infracellular plexus, (Fig. 6e) followed by outgrowth over the emerging dendrites, the place of the supranuclear capuchon, the stage of the young climbing fiber arborization, and finally its adult form (Fig. 6h). The shift of the climbing fiber synaptic connections with the filopodia of the Purkinje cell soma, to their position on stubby spines on the smooth proximal dendrites of the Purkinje cells and their replacement by the inhibitory synapses of the basket cell axons, was documented in the electron microscopic studies of Larramendi [69] and Morara

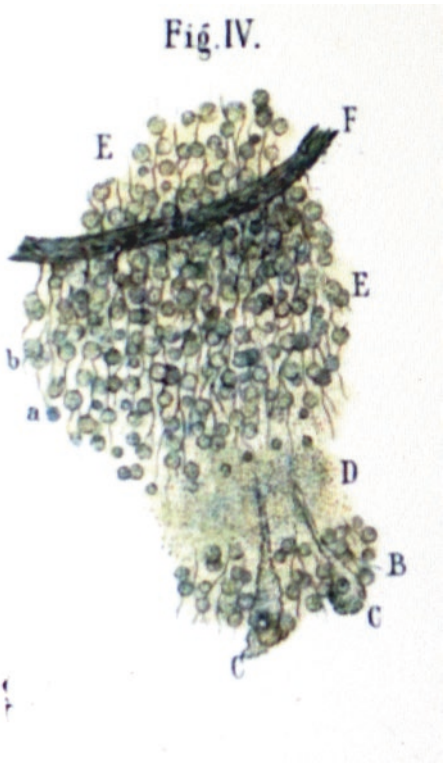

Fig. 7 Cortex of neonate dog showing the stratum granulosum peripheric um. Carmine staining (Reproduced from Hess [50]). *Abbreviations*: *a* granule cell with processes directed at the periphery, *B* stratum granulosum centrale, *b* granule cell with filiform processes at both ends, *C* nerve (Purkinje) cells, *D* stratum moleculare, *E* stratum granulosum peripheric um, *F* pia mater

et al. [82]. Multiple innervation by climbing fibers of the Purkinje cell was noticed by Cajal and others (Fig. 6e, h). The elimination of redundant climbing fibers was shown much later in physiological studies, reviewed by Hashimoto and Kano [46].

The external granular layer (EGL) of the cerebellar anlage gives rise to the granule cells, although, during its history of more than 150 years, it was supposed to contribute to each cell type of the cerebellum. The first description of the EGL dates from Hess [50], who illustrated it as the stratum granulosum peripheric um in the cortex of a neonate dog (Fig. 7). Its cells are provided with radially oriented filiform processes. In due time, the layer disappears, leaving only a few cells near the pia mater. Obersteiner [90] distinguished a superficial, tightly packed, and a deep layer with loosely arranged rounded cells in the EGL (Fig. 8). Like Hess, radial processes in the molecular layer were found to originate from these cells. Later authors often referred to the EGL as "Obersteiner's layer." Schaper [102] in fish and Herrick [49] in mice and guinea pig observed the origin of the EGL from the ventricular matrix next to the caudal attachment of the roof plate of the fourth ventricle and its rostral migration over the cerebellar surface. They observed mitoses in the superficial EGL and identified it as a secondary matrix. Miale and Sidman [81] dated the origin of the EGL in the mouse at E13, when the generation of Purkinje cells has ceased and found that the proliferation in the EGL lasts till the

Fig. 8 Section through the cerebellar cortex of a neonate. Carmine staining *1* upper layer of the EGL (Basalschichte), *2* second granular layer, *3* molecular layer (radiär gestreifte Schichte), *4* Purkinje cell layer (tangentielle Schichte), *5* permanent granular layer (Reproduced from Obersteiner [90])

third postnatal week. Proliferation in the EGL is regulated by sonic hedgehog, secreted by the subjacent Purkinje cells [30].

In his 1890a paper, Cajal described different cell types in the EGL and the molecular layer (Fig. 9). Horizontal, bipolar neurons, with horizontal axonal expansions extending in the length of the cerebellar folia, occur in the deep layer of the EGL. Bipolar neurons with radially oriented processes occur in the molecular layer (Fig. 9). Strange as it may seem to us now, Cajal did not recognize these neurons as stages in the migrating granule cells, at least, with his scientific rigor, he judged that he had too little material to draw this conclusion. Later he identified the origin of the parallel fibers from the horizontal bipolar neurons, the emergence of a third, protoplasmic process, and the translocation of the nucleus in this process through the molecular layer into the internal granular layer (Fig. 10). Here its rounded cell body bears multiple dendrites most of which are resorbed when it settles deep in the granular layer in regions were the mossy fiber rosettes have attained their adult form (Fig. 10) [25]. The parallel fibers are stacked from the bottom of the molecular layer upward. A similar gradient as present for the differentiation of the Purkinje cells in different lobules of the cerebellum was found for the differentiation of the granule cells [3].

Granule cell precursors use Bergmann glial fibers for their migration [97]. These fibers, with their typical lateral processes and their attachment to the meningeal surface

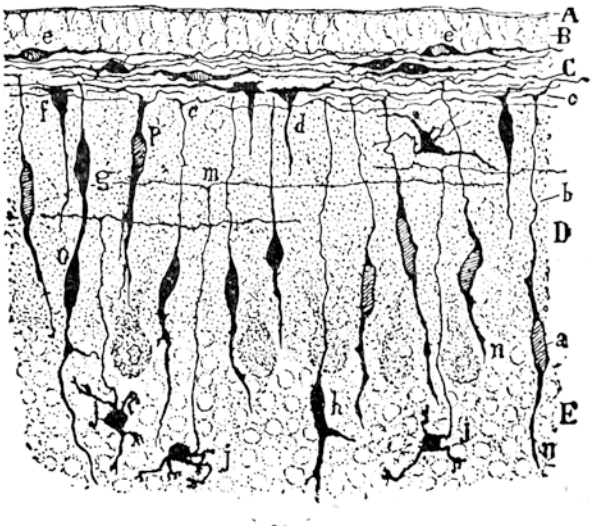

Fig. 9 Neurons in the developing EGL and the molecular layer (Reproduced and relabeled from Cajal [24]). *Abbreviations*: *A* cuticula, *a* vertical bipolar cell, *b* ascending process that terminates in c. in a bifurcation, *B* layer of epithelial cells, *C* zone of horizontal bipolar cells, *d* transitional cell that resembles a horizontal bipolar neuron, *D* molecular layer, *E* granule cell layer, *e* horizontal bipolar cells, *g* parallel fiber, *j* granule cell, *m* bifurcation of granule cell axon, *n* descending process of a vertical bipolar cell

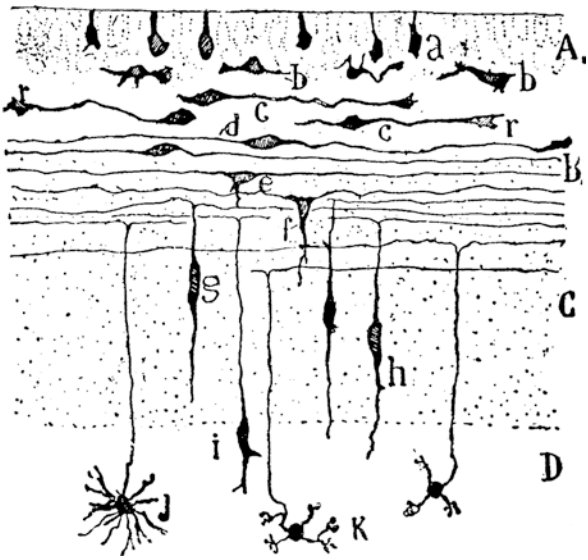

Fig. 10 Stages in the development of the granule cells (Reproduced from Cajal [25]). *Abbreviations*: *A* external granular layer, matrix, *B* external granular layer, layer of horizontal bipolar cells, *b, c, d* horizontal bipolar cells, *C* molecular layer, *D* granular layer, *e, f* bipolar cells with radial process, *g, h* vertical bipolar cells, *j* granule cell with multiple dendrites, *k* adult granule cell, *r* growth cone of parallel fiber

of the cerebellum, were described by Bergmann [13]. Bergmann glia have been described as originating from the Golgi epithelial cells by translocation of their cell bodies to the Purkinje cell layer [125] but have also been traced from cells proliferating in the cerebellar white matter [126]. The orientation of the parallel fibers clearly is established very early as processes of the horizontal bipolar cells in the EGL. Purkinje cell dendritic arbors derive their plane shape and their orientation perpendicular to the parallel fibers from the interaction with these fibers during their development [3, 84]. However, the orientation of the parallel fibers in the long axis of the folia can be uncoupled after perinatal administration of methylazoxymethanol in rats [51].

Development of the Cerebellar Nuclei

The first study of the development of the cerebellar nuclei in different classes of vertebrates is by Rüdeberg [100]. In the tradition of Bergqvist and Källén [14], he traced the origin of the cerebellum from two, subsequent migration areas, A and B, from the ventricular neuroepithelium of the dorsal column of the first rhombomere. The dorsal part of the first migration area A gives rise to the external granular layer; its middle portion A_2 merges with part of the second migration area B into the cell group A_2B; its dorsal part, A_1, develops outside the cerebellum, into the isthmic nucleus. The dorsal part of migration B gives rise to the Purkinje cell layer (Fig. 11). In birds cell group, A_2B develops into the cerebellar nuclei, and in mammals, it gives rise to the lateral (dentate) nucleus. The interposed and fastigial nucleus stem from ventral parts of migration B. The development of the cerebellar nuclei in Cetacea follows the same pattern [63]. According to Korneliusen [62], all nuclei in the rat develop from the deep layer of migration B. The nomenclature used by Feirabend [36] for the early development of the chicken cerebellum is different, but his account of the origin of the cerebellar nuclei from the ventricular zone is very similar to that of Rüdeberg. The two migration layers were also recognized by Altman and Bayer [4–6] in the rat. The first migration layer, with exception of its ventral portion (Rüdeberg's A_1), gives rise to all cerebellar nuclei and was indicated as the nuclear transitory zone (NTZ). A second migration layer (Rüdeberg's B) gives rise to the Purkinje cells. As a consequence, the future Purkinje cells migrate through the NTZ to reach their superficial position. The NTZ splits in a dorsomedial group of longitudinally oriented cells and a superficially located lateral group with a transverse orientation (Fig. 12). The latter migrates medially and gives rise to axons that cross in the cerebellar commissure forming the uncinate tract that takes origin from the fastigial nucleus. The superficial location and the origin of the uncinate tract from this nucleus and its migration to a more ventral position were experimentally verified by Bourrat and Sotelo [17] (Fig. 12, inset). The longitudinally oriented neurons will develop into the interposed and lateral (dentate) nuclei. With the demonstration by Machold and Fishell [77] and Wang and Zoghbi [119] that glutamatergic neurons of the nuclei are derived from the upper rhombic lip, Rüdeberg's migration A, or Altman's nuclear transitory zone, became a layer of tangentially migrating neurons destined for the cerebellar nuclei.

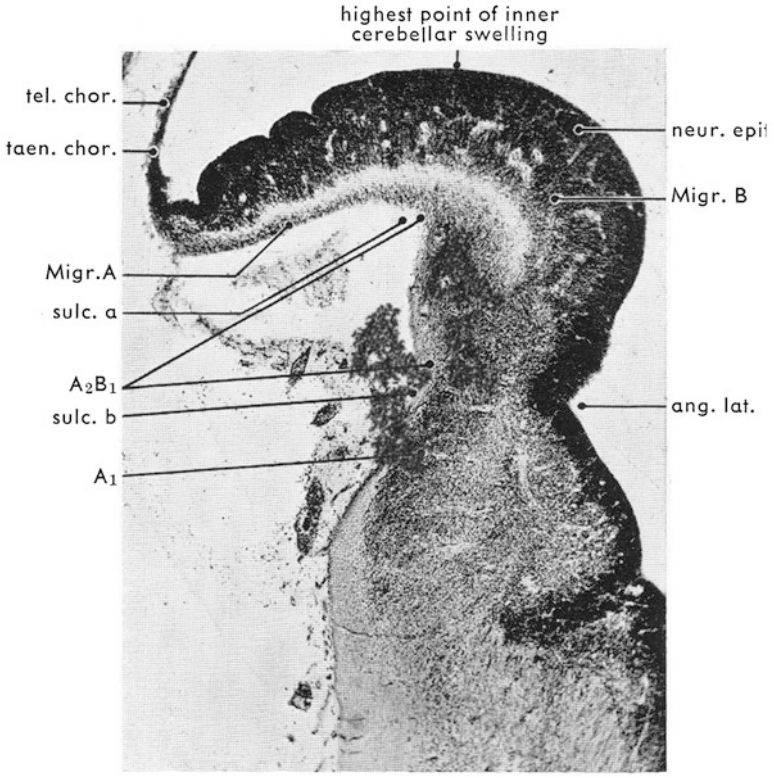

Fig. 11 Transverse section of the cerebellar anlage from a 21 mm human embryo (Reproduced from Rüdeberg [100])

Development of Longitudinal Purkinje Cell Zones

Longitudinal Purkinje cell zones are among the first features of the cerebellum to develop as discrete multicellular clusters that will extend rostrocaudally as adult, monolayered zones. Purkinje cell zones were first identified by their projections to cerebellar and vestibular target nuclei and their afferent olivocerebellar fibers occupy [113, 114], illustrated in Fig. 13a–c. It should be noticed that the B zone (green) and the C1, C3, and Y zones (red) are restricted to the anterior lobe and the simplex lobule, and to lobule VIII and its hemisphere, the copula. Other zones extend over most of the rostro-caudal length of the cerebellar surface. Their development has been studied in serial, Nissl-stained sections in different species and by using Purkinje cell-specific markers. Their development was first studied by Korneliusen [61] in Cetacea. In *Balaenoptera musculus* (blue whale) and *Balaenoptera physalus* (fin whale) embryos, he distinguished four Purkinje cell clusters in the cortical anlage, each cluster being topographically related to one of the incipient cerebellar nuclei (Fig. 14). Clusters are clearly demarcated and differ

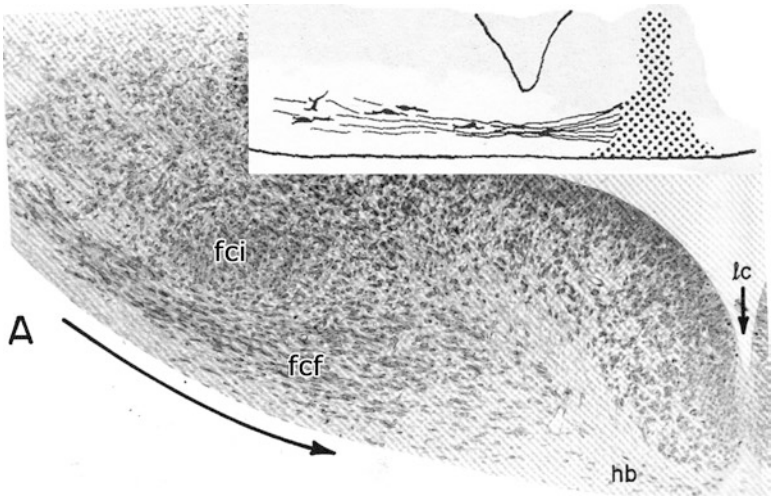

Fig. 12 Transverse section through the cerebellar anlage of a E17 rat embryo, showing the division of the nuclear transitory zone in a group of medially migrating, future fastigial nuclear neurons (*fcf*) that will give rise to the uncinate tract (*hb*), and a group of longitudinally oriented neurons (*fci*), the source of the interposed and lateral nuclei (Reproduced from Altman and Bayer [5]. *Inset*: Injection of horse radish peroxidase (stippled area) labels fibers of uncinate tract in the cerebellar commissure and cells of contralateral fastigial nucleus in a E16 rat embryo [17])

in the degree of differentiation of their cells. Raphe-like, cell-poor differentiations within the medullary substance demarcate the borders between the cluster/nuclear complexes. Clusters extend all over the length of the still smooth cerebellar surface. Three subdivisions are present in the medial cluster overlying similar differentiations within the medial nucleus. A narrow medial intermediate cluster is related to the small anterior interposed nucleus, the wide lateral intermediate cluster to the large posterior interposed nucleus, and the lateral cluster is topographically related to the anlage of the lateral cerebellar nucleus. A very similar clustering in the incipient cortex was found in the rat [62] (Fig. 14). The same relations of the cerebellar nuclei were found as in whale embryos, but the lateral intermediate cluster, like its target nucleus, the posterior interposed, is smaller and of the same size as the medial intermediate zone and the anterior interposed nucleus. In the rat, a small, additional X zone was present between the lateral and lateral intermediate zone, related to the dorsolateral hump of the anterior interposed nucleus. The medial intermediate and the X clusters are partially covered by the adjoining clusters. Four Purkinje cell clusters were identified by Feirabend [36] in chick embryos. In later stages, migrating strands of granule cells ("granule cell raphes," Fig. 15) are located between and within the clusters, subdividing them in smaller units. The existence of such a second generation of clusters has not been confirmed, but the Purkinje cell raphes have also been identified in mammals and have been used to delineate Purkinje cell clusters and zones in histochemical studies [59, 60, 75, 98]. Cerebellar zonation in early postnatal avian stages was documented by Braun et al. [20].

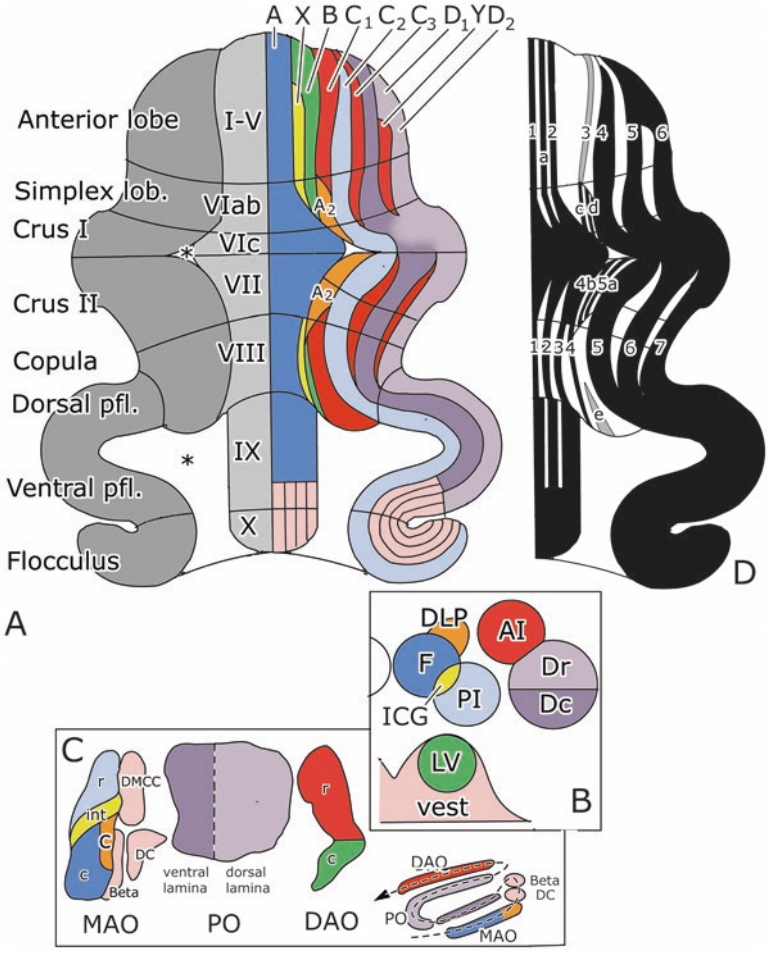

Fig. 13 (**a**) Diagram of the Purkinje cell zones in a flattened map of the cortex of the cerebellum of the rat. The cerebellar and vestibular target nuclei of the zones are indicated in (**b**), the source of their climbing fiber afferents in a flattened map of the inferior olive in (**c**). A map of the distribution of zebrin-positive (*black*) and zebrin-negative Purkinje cells is illustrated in (**d**). Zebrin-positive zones are numbers 1–7. Note that the Purkinje cells of the B, C1, C3, and Y zones are zebrin negative

Purkinje cell clusters have been identified during the development of the primate cerebellum. The first illustrations in human fetuses can be found in Langelaan [67] and Hochstetter [54]. They were studied in macaque monkey fetuses by Kappel [58]. She distinguished two sets of clusters. Those destined to develop in the adult A, C2, and D1 and D2 zones reach the still smooth surface of the cerebellum early (Fig. 16). The clusters that will give rise to the future B, C1, and C3 zones reach the surface later. For some time, they are still partially covered by the neighboring clusters, a phenomenon also noticed for the same clusters in Korneliussen's [64] paper on the rat corticogenesis. Korneliusen's medial and lateral intermediate and his X zone clearly correspond to the monkey C1, C2, and C3 zones, respectively. In the

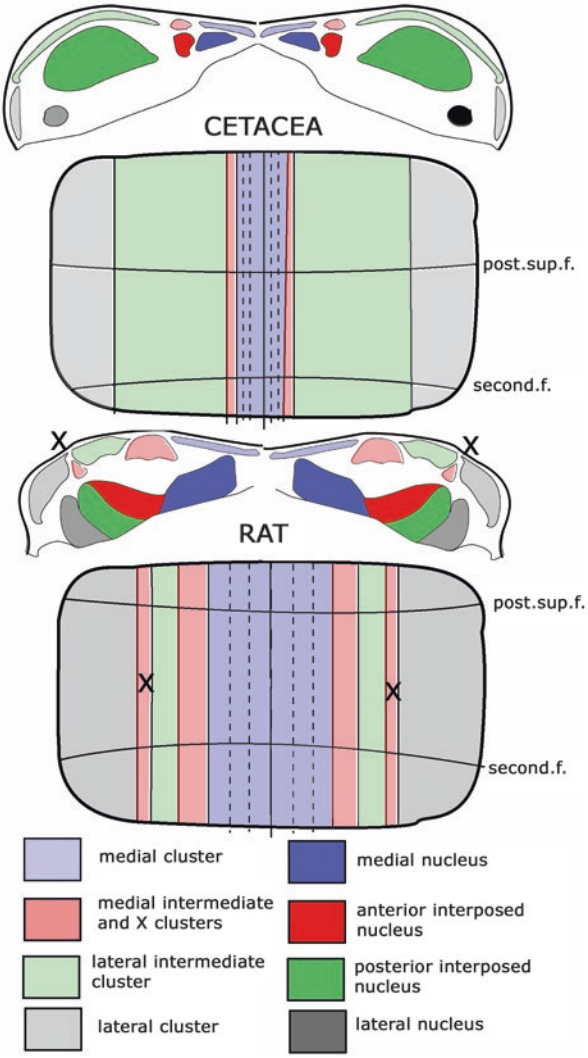

Fig. 14 Transverse section and a diagram of the flattened cerebellar cortex showing Purkinje cell clusters in a 30 mm cr rat embryo and a 17 cm cr *Balaenoptera physalus* embryo (Modified from Korneliusen [61, 62])

monkey fetus, cell strands connect the C1 and C3 clusters with the anterior interposed nucleus (Fig. 17). The same Purkinje cell clusters also can be recognized in human fetuses, where the large size of the lateral D cluster should be noticed (Fig. 18). The differentiation of the human dentate nucleus in a dorsomedial portion with an early differentiating coils, and a late developing ventrocaudal part, was first described by Weidenreich [124]. The general conclusion of these studies is that Purkinje cell clusters transform directly into the adult pattern of Purkinje cell zones.

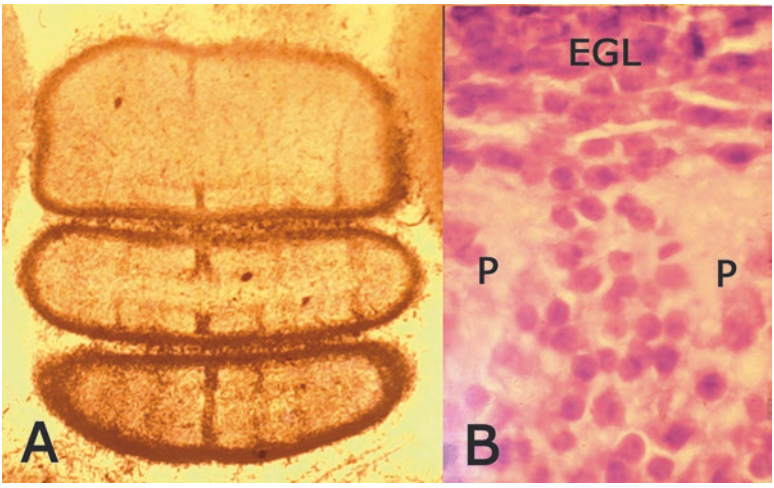

Fig. 15 Granule cell raphes in a 14-day chick embryo. (**a**) Loyez stain of anterior lobe, the EGl, and the granule cell raphes are stained. (**b**) Nissl stain. *EGL* external granular layer, *P* Purkinje cell clusters (Courtesy Dr. Hans Feirabend)

Nothing is known about the development of the detailed (somato) topical patterns [34] in the Purkinje cell zones.

The role of cadherins, adhesion molecules that play an important role in cerebellar development, was reviewed by Redies et al. [99]. Different cadherins are expressed by Purkinje cell clusters early in chick embryos and provide an adhesive code for parasagittal cell domains in avian and mammalian embryos (Fig. 19) and characterize interconnected grisea, such as Purkinje cell clusters and the cerebellar nuclei [8, 86]. In mice, these cadherin domains resemble the Purkinje cell zones as they are known in rats.

Wassef and Sotelo [120] and Wassef et al. [123] traced the development of Purkinje cell clusters in rats, using markers that are expressed by all adult Purkinje cells. Not all Purkinje cell clusters express these markers during development. Different patterns of labeling were observed for different markers. Whether this is caused by a different phenotype of the immature Purkinje cells or by a difference in time scale of the expression of the different markers is not clear. The number of clusters identified was greater than in previous studies, and, therefore, a comparison with them was not attempted.

Another set of Purkinje cell-specific antibodies was developed by Hawkes and Leclerc ([48]: mapQ-113, zebrin I) and Brochu et al. ([21], zebrin II). The epitope of zebrin II was found to be aldolase C [1]. These antibodies stain a subpopulation of Purkinje cells. Multiple longitudinal strips of zebrin-negative Purkinje cells in the anterior lobe and the simplex lobule, in the posterior cerebellum in the pyramis

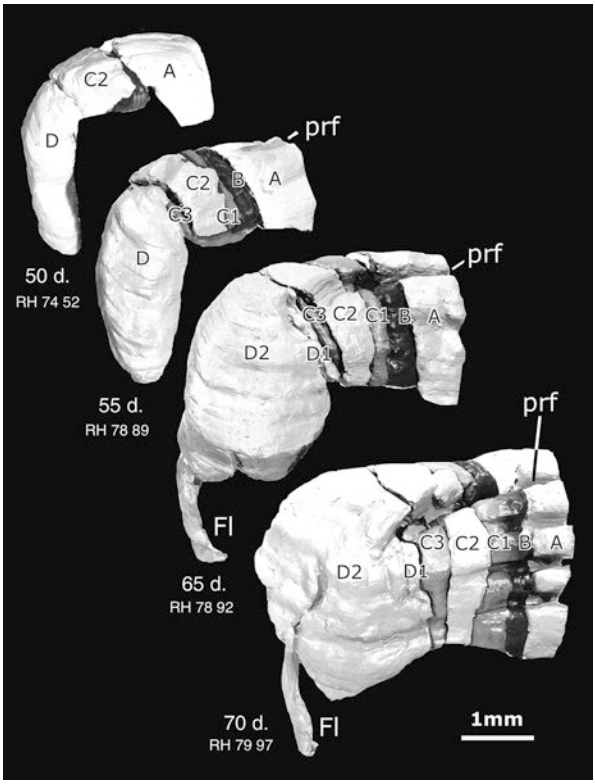

Fig. 16 Photographs of the rostral aspect of reconstructions of the Purkinje cell layer of the cerebellum of four fetuses of the rhesus monkey. Clusters are indicated with different shadings. Note the superficial location of Purkinje cells of the early arriving clusters D, C2, and A in the youngest fetus and the gradual emergence at the surface of the later arriving clusters B, C_1, and C_3. Compare with sections of 55-, 65-, and 70-day-old fetuses in Fig. 16. *Abbreviations*: *Fl* flocculus, *prf* primary fissure (Reproduced from Kappel [58])

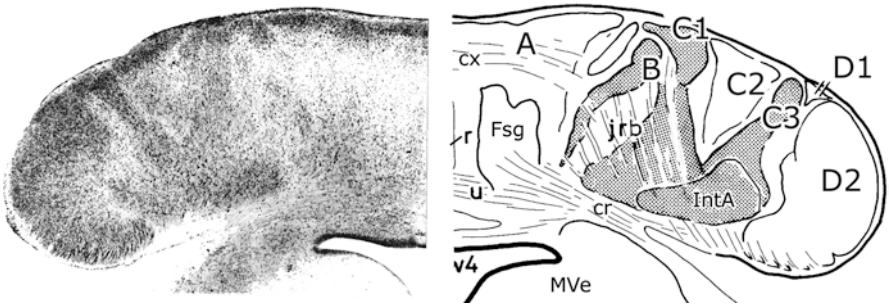

Fig. 17 Coronal section through the cerebellum of a 55-day-old rhesus monkey fetus. Note superficial location of the Purkinje cells of the early arriving clusters A, C2, and D, which still partially cover the later arriving deep clusters B, C_1, and C_3. *Abbreviations*: *cr* restiform body, *IntA* anterior interposed nucleus, *v4* fourth ventricle (Reproduced from Kappel [58])

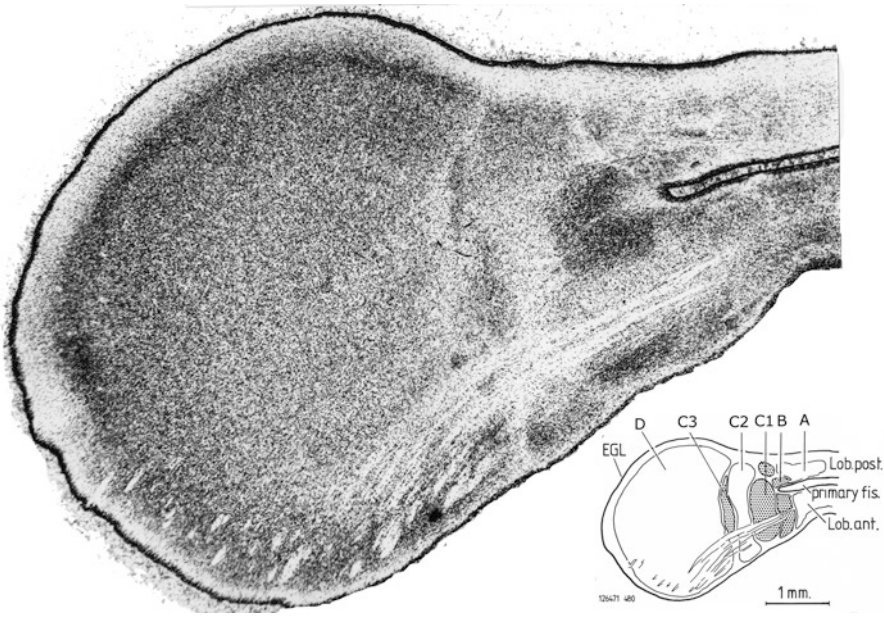

Fig. 18 Purkinje cell clusters A–D in a transverse section of a human 65 mm cr fetus. *EGL* external granular layer

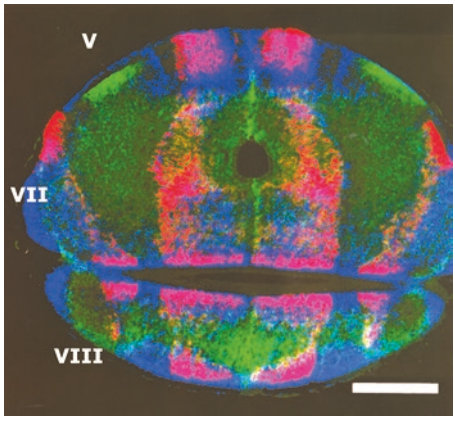

Fig. 19 Zonal distribution of different cadherins in a section through the E11/E12 chicken cerebellum. *Red* Cad6b, *blue* Cad7, *green* R-cadherin. Scale bar 500 µm (Reproduced Arndt et al. [8]

and the adjoining paramedian lobule, separate zebrin-positive strips (Fig. 13d). Expression of the zebrin antigen starts relatively late in P6 rat neonates. At P12, it is present in all Purkinje cells. Subsequently immunoreactivity is selectively suppressed, resulting in the adult-striped pattern [73]. A similar type of development has been found for another late-onset marker for longitudinal zones, heat-shock protein 25 [7]. In studies of the development of the zebrin pattern, bridging the gap

between prenatal clusters and adult zebrin-negative and zebrin-positive strips proved to be difficult [68].

One of the problems is that zebrin-positive and zebrin-negative strips do not map one to one on the Purkinje cell zones defined by their corticonuclear and olivocerebellar identity. The zebrin immunoreactivity of these Purkinje cell zones was established by Voogd et al. [118], Voogd and Ruigrok [117], and Sugihara and Shinoda [107]. Their studies also revealed a number of additional, narrow zebrin-positive strips that were formally discarded as satellite bands. In the rat hemisphere, the B, C1, C3, and Y zones were found to be zebrin negative, the intercalated C2, D1, and D2 zones were zebrin positive (compare Fig. 13a, d). In the vermis, the A zone consists of multiple zebrin-positive and zebrin-negative subzones. Earlier publications on differences in birth date between the Purkinje cells of different clusters [37] were succeeded by the viral labeling studies of Hashimoto and Mikoshiba [47] that showed that Purkinje cells in mice born at E11.5 form clusters that will develop into the zebrin-positive (C2, D1, and D2) zones, whereas Purkinje cells born at E12.5 develop in the zebrin-negative (B, C1, C3, and Y) zones [85] (Fig. 20). Earlier Kappel [58] found these late-born Purkinje cell clusters to arrive later at the cerebellar surface than the early-born clusters. Just as the number of zebrin-positive and zebrin-negative stripes increased in recent studies, the number of Purkinje cell clusters identified in E17.5 mice embryos increased to 54 on each side [39]. These authors traced the development of these clusters into the adult zebrin pattern. More recent developments in this field were reviewed by Arancillo et al. and in Leto et al. [74].

Development of Connections

The development of the afferent climbing and mossy connections of the cerebellum has received more attention than the output systems of the cerebellar nuclei. A closed chapter in the study of the development of cerebellar connections is the study of their myelinization. Axonal systems acquire their myelin sheaths at different pre- and postnatal dates. Myelin-stained sections can provide information on their topography. The method was mostly used in human fetuses and neonates. Like modern MRI tractography. It does not provide information on the precise origin and termination of the tracts nor on the direction of impulse propagation. A good example is the dorsal spinocerebellar tract that bore the eponym Flechsig's tract after its discovery in the myelogenetic studies of this author [38]. The localization of this tract in the restiform body was illustrated by Darkschewitsch and (Sigmund) Freud [31]. In a human fetus, it consists of a core of myelinated cuneocerebellar and dorsal spinocerebellar fibers and an unmyelinated periphery of olivocerebellar fibers (Fig. 21). Details on the intracerebellar topography were published by De Sanctis [32]. The state of the art at the end of the nineteenth century was reviewed by von Bechterew [12].

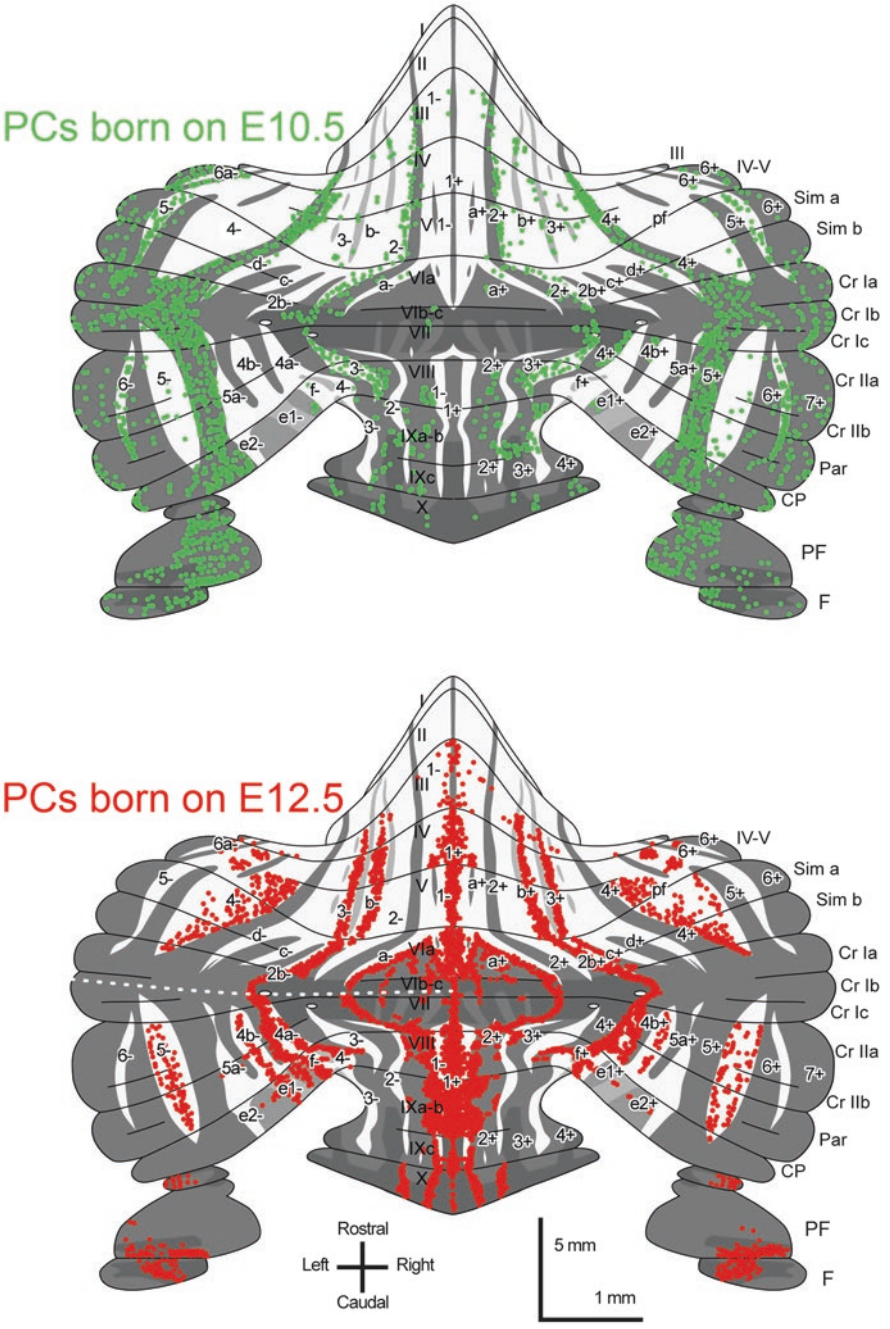

Fig. 20 Distribution of Purkinje cells born on E10.5 and E12.5 superimposed on a map of the zebrin-positive (*gray*) and zebrin-negative strips of the cerebellum of the mouse. Early-born Purkinje cells constitute zebrin-positive bands; late-born Purkinje cells constitute zebrin-negative bands (Reproduced from Namba et al. [85])

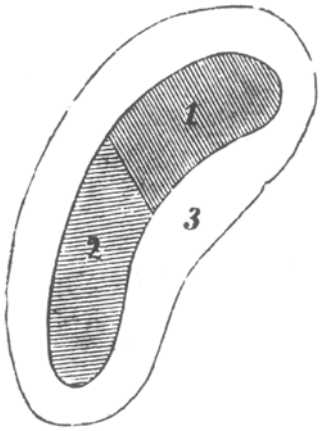

Fig. 21 Diagram of the myelinization of the restiform body in a human fetus. The central, myelinated core consists of cuneocerebellar (*1*) and dorsal spinocerebellar tract fibers (*2*). The unmyelinated periphery consists of olivocerebellar fibers (From Darkschewitsch and Freud [31])

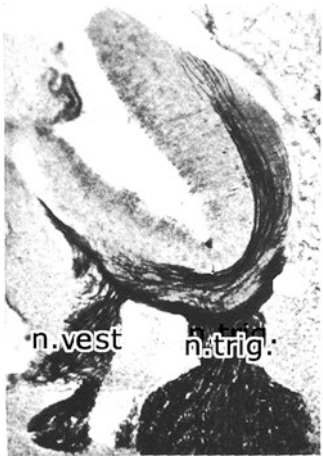

Fig. 22 Sagittal section through an 8 mm mouse embryo; Cajal silver staining. Axons of the ascending branch of the vestibular nerve enter the cerebellar anlage (Reproduced from Tello [108]). *Abbreviations*: *n.trig* trigeminal nerve, *n.vest* vestibular nerve

According to Tello [108], who used the Cajal silver impregnation in mouse embryos, the first system to enter the cerebellum in an 8 mm mouse embryo is the ascending branch of the bifurcating vestibular nerve. These fibers appear to be directed at the caudal pole of the cerebellar anlage, where some will cross the midline (Fig. 22). At a later stage, another afferent system, Tello's faisceau bulbo- or olivo-cérébelleuse, enters the rostral pole of the cerebellum. Its fibers form the cerebellar commissure which, in a 13 mm mouse embryo, extends over the entire rostro-caudal dimension of the cerebellum (Fig. 23). Tello's observations on the early arrival of primary vestibulocerebellar fibers were confirmed by Morris et al. [83], using the parvalbumin immunoreactivity of these fibers in rat embryos. First the fibers are located immediately under the pial surface. Later they are found in medially and caudally directed bundles that will reach the granular layer of the uvula-nodulus. These fibers may serve as pathfinding axons for non-immunoreactive

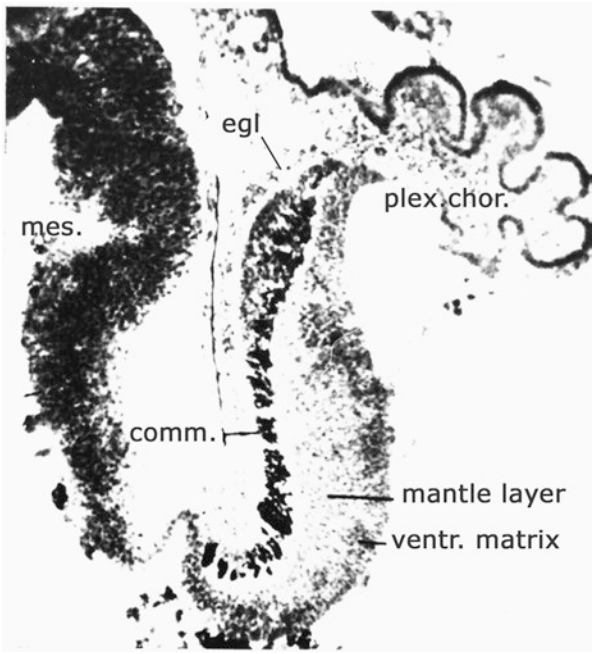

Fig. 23 Sagittal section through a 13 mm mouse embryo; Cajal silver staining, showing the cerebellar commissure (Reproduced from Tello [108]). *Abbreviations*: *comm* cerebellar commissure, *egl* external granular layer, *mes* mesencephalon, *plex. chor.* choroid plexus, *ventr. matrix* ventricular matrix

fibers, possibly belonging to secondary vestibulocerebellar fibers from the vestibular nuclei. The development of differential projections of cristae and maculae in mice to the uvula-nodulus was studied by Maklad and Fritsch [78].

Of the other mossy fiber afferent systems, the development of the spinocerebellar projection has received most attention. The bilateral, regular collateralization of spinocerebellar fibers that form multiple parasagittally oriented terminal fields in the granular layer was first described in our lab for mammals [114] and birds [112]. Lakke et al. [66] traced spinocerebellar axons with WGA HRP in chicken embryos. They enter the rostral cerebellum in Tello's bulbocerebellar fascicle at the seventh incubation day. They course superficially, to enter the cerebellar commissure 2 days later. The bundle of spinocerebellar axons gives off collaterals which enter the Purkinje cell clusters, from where they extend into the molecular layer (Fig. 24). Spinocerebellar fibers disappear from the molecular layer, and terminal rosettes in the inner granular layer develop late before and after hatching [91]. In mammals, a similar sequence is present in the development of the spinocerebellar pathway. Their early entrance in the rostral cerebellar anlage in E13 mouse embryos, their superficial location, and their decussation in the cerebellar commissure are observed at E15. No parasagittal arrangement is visible at E19 [43]. According to Arsénio

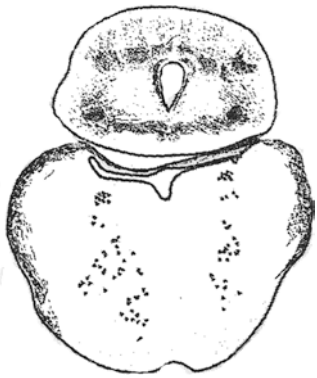

Fig. 24 Bundles of spinocerebellar fibers in an 11-day incubation chick embryo are positionally related to the Purkinje cell clusters. Collaterals are seen to enter these clusters (Reproduced from Lakke et al. [66])

Nunes and Sotelo [9], the columnar distribution of spinocerebellar fibers in the rat develops postnatally from a more diffuse stage that was not observed in birds. A distinct topographical relationship of these columns to the zebrin pattern, i.e., to the Purkinje cell zones, was described by Ji and Hawkes [55]. According to these authors, despite the early zonal distribution of the mossy fibers being dependent on Purkinje cell clustering, granule cell-mossy fiber interactions if disturbed by chemical ablation of the EGL result in blurring of this pattern [56].

Little is known about the development of other mossy fiber systems. For the development of the pontocerebellar projection, Bechterew [11] made an interesting observation. He found an early myelinating "spinal system" in the brachium pontis of human neonates that can be traced from the caudal pontine nuclei and the nucleus reticularis tegmenti pontis into the flocculus and the anterior cerebellum. The "cerebral system" of the brachium pontis, which courses from the rostral pontine nuclei to the posterior cerebellum, is still unmyelinated at the time (Fig. 25). This is in accordance with more recent observations that the main projection of the caudal pontine nuclei, which receive their afferents from motor cortical areas, is to the anterior lobe, whereas rostral pontine nuclei that are innervated by cortical association areas mainly project to the caudal cerebellum [115]. Tolbert and Panneton [110] described transient extra-pontine cerebrocerebellar connections from the somatosensory cortex to the cerebellar cortex and nuclei in kittens using axonal transport of tritiated amino acids, horseradish peroxidase, or fluorescent dyes. These projections arise as collaterals from the pyramidal tract, passing through the superior cerebellar peduncle to terminate in the nuclei, and caudal to the pons, bilaterally through the restiform body to be distributed as mossy-like fibers to the granular layer of the anterior lobe, the lobulus simplex and the paramedian lobule. The projections to nuclei and cortex are somatotopically organized [94, 109]. Nuclear projections are present at P6–P8, cortical projections between P8 and P10. After the seventh postnatal week, no cerebrocerebellar projections were present any more. Earlier, a similar transient pathway from the occipital region of the hemisphere to the paraflocculus was observed in neonatal rabbits [33].

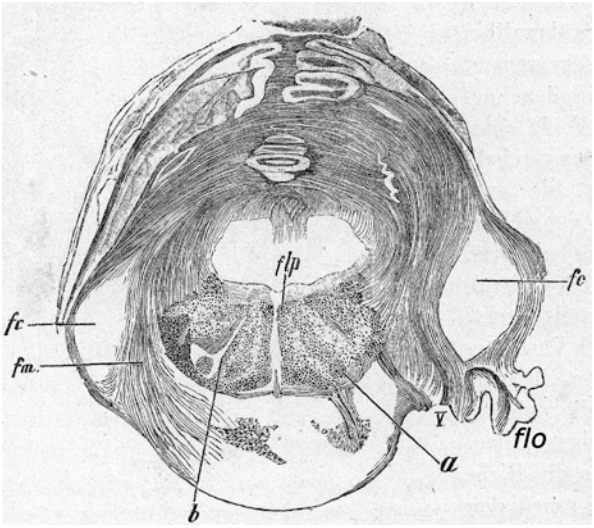

Fig. 25 Transverse myelin-stained section through the brainstem and cerebellum of a human neonate (Reproduced from Bechterew [11]). *Abbreviations*: *a* central tegmental tract, *b* superior olive, *fc* cerebral system of the brachium pontis, *flo* flocculus, *flp* medial longitudinal fascicle, *fm* spinal system of the brachium pontis

Since the studies of Voogd [114] and Groenewegen and Voogd [44], it is known that the topographical organization of the olivocerebellar projection closely matches the longitudinal zonal organization of the corticonuclear projection and their localization in white matter compartments. Therefore the question is not whether it is likely that Purkinje cell clustering determines this pattern but rather how this is achieved. Olivocerebellar fibers enter the cerebellum early, in E8.5–E9 chick embryos, presumably, in Tello's olivocerebellar bundle; initial target selection occurs at E10. Affinity of Purkinje cell clusters for the olivocerebellar fibers from particular subdivisions of the inferior olive was shown by Chédotal and Sotelo [26], Wassef et al. [121, 122], and Paradies et al. [92] (Fig. 26). The cell adhesion molecule BEN was found to be present in subdivisions of the inferior olive and Purkinje cell clusters. However, non-BEN i.r. clusters also were found to receive BEN-ir olivocerebellar fibers ([28]; Pourquié et al. [96]). Irrespective of reversal of the cerebellar plate, olivocerebellar fibers recognize polarity cues in their target region that organize their antero-posterior topography [27]. Ephrins and their receptors are distributed in parasagittal domains in chicken embryos [60]. These domains were found to correspond to the olivocerebellar mapping domains [89].

Although the development of corticonuclear connections was implicit in some of the cited papers on the development of longitudinal Purkinje cell zones, the subject has received little attention. The uncinate tract, as the main efferent system of the fastigial nucleus, was considered in section "Development of the

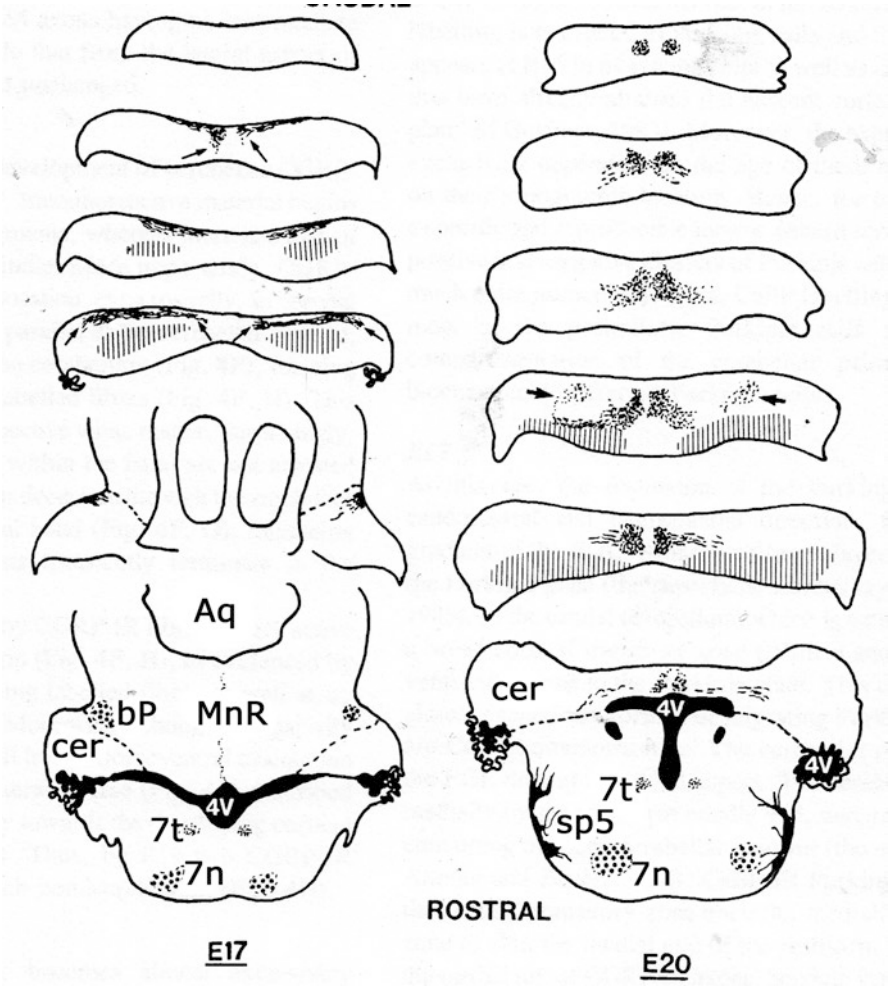

Fig. 26 Location of CGRP immunoreactive olivocerebellar fibers in the cerebellum of an E17 and an E20 rat embryo. CGRP immunoreactive brain stem nuclei and tracts are indicated. Cerebellar nuclei are hatched (Reproduced from Chédotal and Sotelo [26]). *Abbreviations*: *4V* fourth ventricle, *7n* facial nucleus, *7t* genu facial nerve, *Aq* aquaduct, *bp* parabrachial nucleus, *cer* cerebellum, *MnR* median raphe, *sp5* spinal tract trigeminal nerve

Cerebellar Nuclei." The development of the brachium conjunctivum was studied in rat fetuses by Cholley et al. [29]. It emerges from the cerebellar nuclei at E15; at E16, it crosses the midline in Wernekinck's decussation [116]. The olivonuclear pathway was found in a more ventral position to decussate rostral to the main portion of the brachium.

Development of the Folial Pattern

Studies of the development of the folial pattern contributed to our present nomenclature of the cerebellum. Kuithan [65] coined the name sulcus primarius for the first fissure to appear medially in the cerebellum of a 5 cm sheep embryo. Another unnamed, fissure is present at this stage running along the caudal rim of the cerebellar anlage, which we now know as the posterolateral fissure (Fig. 27). Next to appear is the fissure that borders the uvula rostrally (our secondary fissure) followed by the prepyramidal fissure. The name "secondary fissure" was introduced by Smith [105] for "one of the two fundamental clefts which cross the mesial plane (that) have been called the 'fissura prima' and the 'fissura secunda' in reference to their relative importance and precocity." Smith only studied adult specimens and must have derived his ideas about the precocity of these fissures from Kuithan's studies. Kuithan's observations were partially confirmed by Stroud [106] in feline and human embryos. However, before any fissures appeared in the future vermis, Stroud observed a parafloccular sulcus in the hemisphere that separates his "pileum" (our ansiform and paramedian lobulus) from his "paraflocculus." Contrary to his term paraflocculus, Stroud's names for the primary fissure (the furcal sulcus) and the anso-paramedian lobules have not survived.

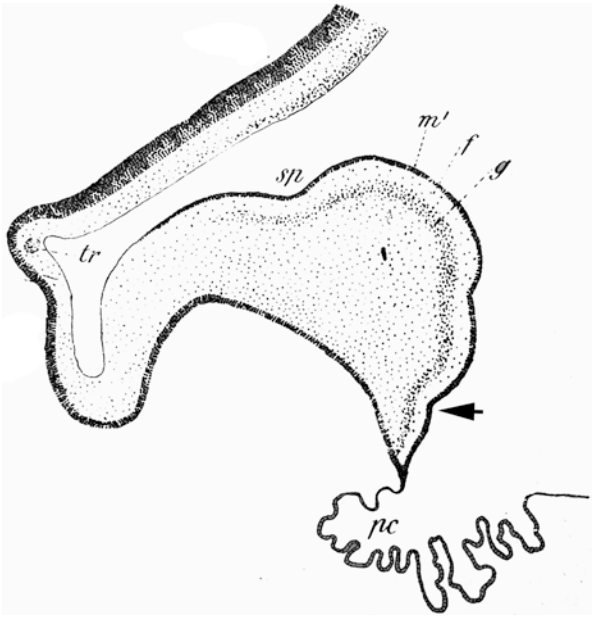

Fig. 27 Sagittal section of a 5 cm sheep embryo, showing the division of the germinal zone of the early cerebellar plate by the primary fissure and an unnamed fissure along the caudal border of the cerebellum (*arrow*) (Reproduced from Kuithan [65]). *Abbreviations*: *f* fibrillar layer, *g* mantel layer, *m′* external granular layer (embryonale Randschicht), *sp.* sulcus primarius, *tr* trochlear nerve

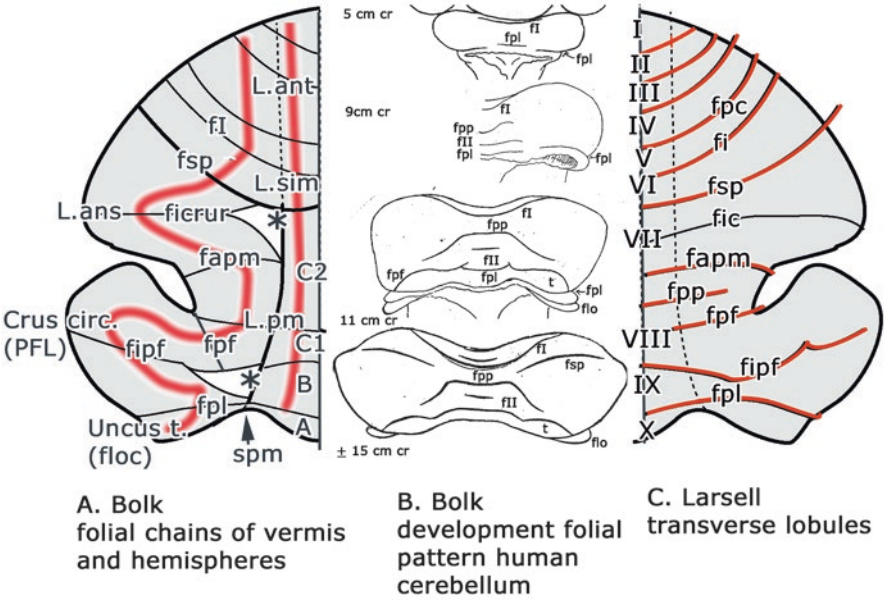

Fig. 28 (**a**) Diagram of Bolk's [16] Bauplan of the mammalian cerebellum. Folial chains of vermis and hemisphere are aligned in anterior lobe and in the pyramis (C1) and the paramedian lobule and behave like independent growth centers in the ansiform lobule-lobule C2 and the paraflocculus-flocculus and lobules A (nodule) and B (uvula) segments (**b**). Drawings of different stages in the development of the human cerebellum (Relabeled from Bolk [16]). (**c**) Larsell's [72] transverse subdivision of the mammalian cerebellum. *Abbreviations*: *A* lobule A (nodulus), *B* lobule B (uvula), *C1* lobule C1 (pyramis), *C2* lobule C2 (folium and tuber vermis), *cop* copula pyramidis, *Crus.circ (PFL)* crus circumcludens (paraflocculus), *Fapm* ansoparamedian fissure, *fI* primary fissure, *Ficrur* intercrural fissure, *fII* fissura secunda, *FLO* flocculus, *fpc* preculminate fissure, *fpf* parafloccular fissure, *fpp.* prepyramidal fissure, *fsp* superior posterior fissure, *L.ans* ansiform lobule, *L.ant* anterior lobe, *L.pm* paramedian lobule, *L.sim* simplex lobule, *PFLD/V* dorsal/ventral paraflocculus, *Spm* paramedian sulcus, *Uncus t. (floc)* uncus terminalis (flocculus)

One of the longstanding controversies in the subdivision of the mammalian cerebellum was whether a subdivision in lobules, separated by transverse fissures, or a sagittal division into vermis and hemispheres was to be preferred. The proponent of the division in vermis and hemispheres was Louis Bolk [16] (Fig. 28a). Bolk's [15, 16] studies of human embryos, which confirmed Bradley's [18, 19] earlier observations, showed that the cerebellum is a compromise between transverse and longitudinal trends in the development of its folial pattern. In the anterior lobe with the simple lobule and in the pyramis with its hemisphere, fissures and lobules of the hemisphere develop as extensions from the vermis. In the ansiform lobule, the paraflocculus and the flocculus fissures develop independently from the vermis (Fig. 28b). The independence of vermis and hemisphere later was emphasized by the local absence of cortex, i.e., of parallel fibers, in these regions [113]. Larsell [70] stated his belief in the prevalence of a transverse lobular subdivision as: "it is clear

in the adult and in the fetus that the lateral parts, namely ansiformis, paraflocculus and the lateral continuation of the pyramis are merely lateral extensions of the medial portions." Larsell identified the posterolateral fissure as the first fissure to develop. It separates the primary divisions of the cerebellum, the flocculonodular lobe, and the corpus cerebelli, from each other. Larsell subdivided the avian and mammalian cerebellum in ten homologous lobules, indicated with roman numerals [71, 72] (Fig. 28c). The development of the folial pattern in birds was also studied by Saetersdal [101]. He agreed with Larsell that the posterolateral fissure is the first to appear but found Larsell's preculminate, prepyramidal, and secondary fissures to appear next. Larsell's preculminate fissure, therefore, represents the true primary fissure, and the lobules of the avian cerebellum should be renumbered.

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The Embryology and Anatomy of the Cerebellum

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Abstract The cerebellum is an important structure in the central nervous system that controls and regulates motor and non-motor functions. It is located beneath the occipital lobe and dorsal to the brainstem. Today, we know much about its complex circuitry and physiology. The cerebellum has a well-defined and highly organized structure. The cortex of the cerebellum contains eight neuronal cell types and receives input from a variety of sites within the CNS and processes the information in a uniform manner. The cerebellum projects to a variety of different sites within the CNS to regulate motor function. Although much has been discovered regarding the complex architecture of the cerebellum, there are significant gaps in our understanding of the broader role of the cerebellum in brain function. In this chapter, we will review briefly the embryological development of the cerebellum and provide an overview of the anatomy of the cerebellum.

Keywords Cerebellum • Embryology • Anatomy • Histology • Function

Introduction

The cerebellum (latin: 'little brain') is located in the posterior cranial fossa and is involved in the regulation of posture, motor coordination, balance, and motor learning. More recently, it has been proposed that it also plays a role in emotion and cognition. The cerebellum consists of a midline region referred to as the vermis, a narrow paravermal area immediately adjacent to the vermis, and large hemispheres on either side. Well-defined fissures divide the cerebellum in a rostral caudal direction into an anterior lobe, posterior lobe, and flocculonodular lobe. The anterior and posterior lobes are divided further, into lobules and folia (in human), which greatly increases the surface area of the cerebellum. The cerebellum consists of a uniform

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layer of cortical grey matter overlying white matter that surrounds four pairs of cerebellar nuclei (CN). The cerebellar cortex consists of three layers: molecular layer, Purkinje cell layer, and granule cell layer. The molecular layer is the outermost layer and is largely a synaptic layer, containing the connections of a number of neurons (e.g., basket and stellate cells) with the dense dendritic arborizations of the Purkinje cells, whose cell bodies are the predominant component of the Purkinje cell layer. The innermost layer of the cortex is the granule cell layer containing Golgi cells, Lugaro cells, unipolar brush cells, and the highly abundant granule cells. Almost all of the neurons of the cerebellar cortex use either the excitatory neurotransmitter glutamate or the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Glutamate is used by the granule cells and unipolar brush cells while the remainder of the cortical neurons use GABA. The CN are primarily composed of large projection neurons that use glutamate as a neurotransmitter and project to nuclei of the thalamus and brainstem. These neurons represent the principal output of the cerebellum. A smaller number of CN neurons are GABA-ergic and project to the inferior olivary nucleus of the medulla. The cerebellum is considered an outstanding model in the research of neurogenesis and circuit assembly because of its well organized structure.

Embryology of the Cerebellum

During prenatal development of the nervous system, the central nervous system originates from the area of the ectoderm known as the neural plate. The neural plate thickens as a result of cell proliferation and then begins to invaginate and thus forms the neural groove. The invagination of the neural groove continues until the lateral edges of the neural groove (neural fold) fuse to form the neural tube through a process referred to as neurulation. As the edges of the neural groove fuse to form the neural tube, which detaches from the ectoderm, a population of the neuroectodermal cells dissociate from the neural fold as the neural crest cells [1]. The rostral extent of the neural tube develops into the prosencephalon, mesencephalon, and rhombencephalon. The prosencephalon undergoes further development to form the telencephalon and diencephalon. The mesencephalon does not undergo further division while the rhombencephalon divides into the metencephalon and myelencephalon. Caudal to the rhombencephalon, the neural tube develops into the spinal cord. The cerebellum develops from the dorsal portions (i.e., the alar plate) of the metencephalon and the neural folds, the latter referred to as the rhombic lips. The alar plate of the rostral metencephalon undergoes bilateral expansion in the dorsolateral region to form the rhombomere 1 (r1). These rostral extensions of alar plate eventually join in the midline to form the vermis of the cerebellum. As the cerebellum begins to form, initially from the dorsal r1, it rotates 90° before fusing at the midline as the vermis [2]. This rotation of dorsal r1 results in the conversion of rostral–caudal axis seen in the early neural tube, into the medial–lateral axis seen in the mature cerebellum (the wing-like bilateral cerebellar primordia) [3]. As the bilateral

cerebellar primordia fuse, the midline vermis is derived from the rostro-medial ends while the cerebellar hemispheres are derived from the more caudo-lateral components of the rhombencephalon [4].

The neurons that reside within the cerebellum are derived from two distinct germinal zones: the ventricular zone and the rhombic lip. The ventricular zone is the neuroepithelium of the alar plate of the rhombencephalon that eventually forms the roof of the fourth ventricle. The neurons derived from the ventricular zone include the Purkinje cells, candelabrum cells, Golgi cells, Lugaro cells, stellate cells, and basket cells. All of these neurons use GABA as a neurotransmitter and reside in the outer two layers of the three layered cortex, except for the Golgi and Lugaro cells of the granular layer [5, 6]. The neurons derived from the rhombic lip use glutamate as a neurotransmitter. This includes the large neurons of the CN (projecting to the diencephalon and brainstem), unipolar brush cells, and granule cells, the most numerous cell in the brain.

Anatomy and Histology of the Human Cerebellum

Functional Divisions of the Cerebellum

The cerebellum is a highly organized structure that is attached to all three components of the brainstem (the midbrain, pons, and medulla) [7]. Fissures divide the cerebellum into three lobes in the rostro-caudal plane. The primary fissure, seen on the superior surface of the cerebellum, separates the anterior lobe from the posterior lobe, while the posterolateral fissure, seen on the inferior surface of the cerebellum, separates the large posterior lobe from the narrow and much smaller flocculonodular lobe. The flocculonodular lobe consists of bilateral extensions of cerebellar cortex called flocculi that are connected to the inferior portion of the vermis called the nodulus. During development, once the anterior and posterior lobes form, smaller lobules begin to form. The lobules undergo further infolding which leads to the formation of folia, which are particularly prominent in human cerebellum. The structure of the folia is consistent throughout the cerebellum, with a three-layered cortex overlying the white matter consisting of the axons projecting to and from the cortex (Fig. 1).

The cerebellum is organized into three functional divisions based on their connections to other brain sites and their respective roles in regulating movement and other non-motor functions. The phylogenetically oldest component of the cerebellum is the flocculonodular lobe. The cortex of this lobe receives input from the vestibular apparatus on the ipsilateral side as well as input from the vestibular nuclei of the brainstem. Therefore, the flocculonodular lobe is commonly referred to as the vestibulocerebellum. The connections of the vestibulocerebellar cortex to the vestibular nuclei are reciprocal, and the cortex of the vestibulocerebellum is the only component of the cerebellar cortex that sends projections directly to sites outside the cerebellum (i.e., the vestibular nuclei of the brainstem) [7]. The vestibulocerebellum participates in the control of balance and eye movements.

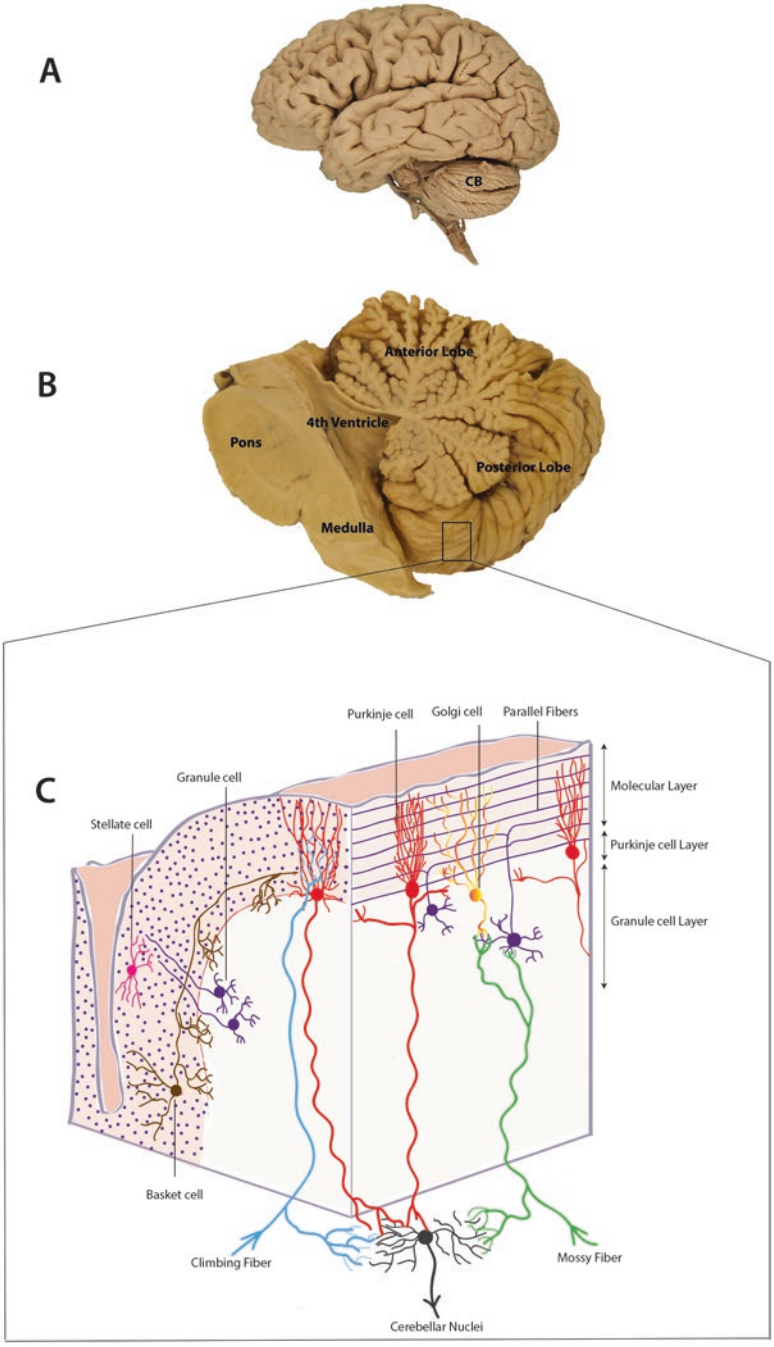

Fig. 1 (**A**) Location of the cerebellum in situ. (**B**) Hemisected view of the cerebellum showing the vermis, the locations of the anterior and posterior lobes, and its anatomical relationship to the brainstem. (**C**) Schematic representation of the cerebellum showing the mossy fibre and climbing fibre inputs to the cerebellar cortex. The mossy fibres contact the granule cells and send collaterals to the cerebellar nuclei while the climbing fibres make contact with the dendrites of the Purkinje cells and may also send projections to the cerebellar nuclei. The granule cells project to the molecular layer and bifurcate to form the parallel fibres that contact the Purkinje cell dendrites as well as the basket cells and stellate cells. The Golgi cells receive input from mossy fibres and also project into the molecular layer of the cortex

The second functional component of the cerebellum consists of the midline vermis of the anterior lobe and a narrow portion of cortex on either side of the vermis referred to as the paravermal cortex. This component is referred to as the spinocerebellum as the bulk of the input to the spinocerebellum is provided by ascending tracts in the spinal cord. The spinocerebellum receives input from the dorsal spinocerebellar tract that transmits proprioceptive, cutaneous, and pressure information from the lower extremity (on the ipsilateral side) [7]. It also receives input from the cuneocerebellar tract, which carries somatosensory information from the upper extremity.

A third major input into the spinocerebellum is the ventral spinocerebellar tract. It transmits information regarding the activity of circuits within the spinal cord involved in regulating motor activity. Additionally, the spinocerebellum also receives inputs from a number of brainstem nuclei including the reticular formation. The spinocerebellum participates in regulating axial and proximal limb muscle musculature involved in balance, posture, and locomotion.

The third and largest functional component of the cerebellum is the pontocerebellum (also referred to as the cerebrocerebellum). It is also the phylogenetically newest component of the cerebellum. It consists of the large hemispheres immediately lateral to the spinocerebellum and receives input principally from the contralateral cerebrum, via the pons. Descending corticopontine fibres from widespread areas of the cerebral cortex (particularly frontal and parietal lobes) project to pontine nuclei of the basilar pons [7]. The neurons of these nuclei send their projections across the midline to project to the cortex of the pontocerebellum. The pontocerebellum is particularly well developed in higher mammals and participates in regulating the coordination of the distal limb musculature as well as playing a role in motor learning.

Cerebellar Cortex

The cortex of the cerebellum is remarkable in its uniformity and segregates into three layers: the outer molecular layer, the Purkinje cell layer, and the inner granule cell layer [6]. The molecular layer contains stellate cells and basket cells but is dominated by the dendrites and axons of other neurons. The molecular layer receives input from neurons of the inferior olivary nucleus of the medulla, and these fibres are referred to as climbing fibres. The climbing fibres make abundant excitatory synaptic connections with the proximal dendritic tree of Purkinje cells [8]. The molecular layer also receives abundant excitatory input from the granule cells of the cerebellar cortex. Granule cells send their axonal projections to the molecular layer cortex where the axons bifurcate and form parallel fibres that run parallel to the cortical surface and make synaptic connections with the dendritic tree of numerous Purkinje cells. The stellate cells of the molecular layer are inhibitory interneurons that use GABA as a neurotransmitter, and these cells are located primarily in the outer part of the molecular layer. These cells also receive input from parallel fibres

and make synaptic contacts with the dendritic tree of Purkinje cells. Finally, the basket cells of the molecular layer also use GABA as a neurotransmitter and are located in the inner portion of the molecular layer. Basket cells receive excitatory input from the parallel fibres of the granule cells and make abundant inhibitory connections on the cell bodies of Purkinje cells in a basket-like manner.

The Purkinje cell layer consists of the large cell bodies of the Purkinje cells, which send an extensive dendritic tree into the molecular layer, and candelabrum cells. The dendritic tree of a single Purkinje cell receives excitatory inputs from a single climbing fibre of the inferior olivary nucleus and numerous inputs from parallel fibres of the granule cells. The Purkinje cell is of particular importance because it represents the sole output of the cerebellar cortex. It uses GABA as a transmitter and projects almost solely to the CN. The exception to this rule is the Purkinje cells of the vestibulocerebellum that also project to the vestibular nuclei of the brainstem. Interspersed between the Purkinje cells within this layer are candelabrum cells that are also GABA-ergic neurons that send their dendritic projections into the molecular layer. The functional significance of these cells is poorly understood.

The granule cell layer is the innermost layer of the cortex and consists of granule cells, Golgi cells, unipolar brush cells, and Lugaro cells. The granule cells are the most abundant neuron in the human nervous system and are packed tightly within the granule cell layer. They receive excitatory input from mossy fibres, which are the principal input into the cerebellum. Mossy fibres originate from numerous sites within the nervous system, including pontine nuclei, nuclei of the reticular formation, vestibular nuclei, and the fibres of the spinocerebellar tracts of the spinal cord. The granule cells, which use glutamate as a neurotransmitter, extend their axons into the molecular layer where they bifurcate into the aforementioned parallel fibres and connect with the dendritic tree of up to hundreds of Purkinje cells. The activity of the granule cells plays a critical role in determining the activity of the Purkinje cells. Additionally, the parallel fibres of the granule cells also shape the activity of other cell types of the cerebellar cortex, including Golgi, stellate, and basket cells. The Golgi cells are relatively large cells that are more abundant in the superficial portion of the granule cell layer, nearer to the Purkinje cell layer [9]. These are also GABA-ergic neurons and extend their dendrites into the molecular layer where they receive synaptic input from the parallel fibres of the granule cells. The Golgi cells also make synaptic connections to the granule cell dendrites, thereby providing a source of inhibition to the granule cell. Unipolar brush cells are neurons within the superficial part of the granule cell layer and like granule cells use glutamate as a neurotransmitter. These cells are more abundant in the vestibulocerebellum than other parts of the cerebellum and are closely associated with mossy fibres and project to granule cells and other unipolar brush cells. The final cell intrinsic to the cerebellar cortex is the Lugaro cell. These are GABA-ergic neurons found primarily in the superficial portion of the granule cell layer. Their dendrites may extend into the molecular layer, while their axon is restricted to the granule cell layer where they make connections with Golgi cells.

Within the cerebellar cortex, the connections and links between the parallel fibres of granule cells and the dendrites of inhibitory cells such as Purkinje cells and

others, and the connections between the mossy fibres and Purkinje cells (and other neurons), make a unique and uniform microcircuitry observed with great consistency in all parts of the cerebellar cortex.

Cerebellar Nuclei (CN)

There are four pairs of CN embedded within the white matter of the cerebellum (dentate, emboliform, globose, and fastigial) that receive input from the cerebellar cortex as well as the collaterals of fibres projecting to the cerebellar cortex [10]. The first cerebellar neurons generated are neurons of the CN. These cells originate from the rhombic lip and migrate tangentially to the nuclear transitory zone (NTZ). The CN constitute the sole output of the cerebellum (excepting some of the Purkinje cells of the vestibulocerebellum), and they receive the output of the cerebellar cortex from the inhibitory Purkinje cells. In addition to the inhibitory inputs from the Purkinje cells, the CN receive the collateral excitatory inputs from mossy fibres and climbing fibres projecting to the cortex. The majority of CN neurons are excitatory neurons that project to sites outside the cerebellum, including the thalamus, red nucleus, reticular formation, and vestibular nuclei. However, a small population of CN neurons are GABA-ergic, and these neurons project to the inferior olivary nucleus.

The fastigial nucleus is the smallest and most medial of the CN. The neurons of this nucleus receive input from the Purkinje cells of the vestibulocerebellum (i.e., flocculonodular lobe). In addition, the fastigial nucleus also receives input from Purkinje cells of the vermis that receive input from the vestibular apparatus either directly or indirectly via the vestibular nuclei. The neurons of the fastigial nucleus project to the brainstem vestibular and reticular nuclei. As mentioned previously, some of the Purkinje cells of the flocculonodular lobe also send direct (inhibitory) projections to brainstem vestibular nuclei.

Lateral to the fastigial nuclei are the globose and emboliform nuclei, also referred to collectively as the interposed nuclei. These nuclei receive input from the Purkinje cells of the vermis and paravermal areas of the anterior lobe of the cerebellum, which in turn receives input from the cuneate nucleus (via the cuneocerebellar tract) and the accessory cuneate nucleus and Clarke's nuclei (via the dorsal spinocerebellar tract). The interposed nuclei send projections primarily to the red nucleus of the midbrain and the ventrolateral nucleus of the thalamus. The latter nucleus relays this information to the primary motor, supplementary motor, and pre-motor cortices of the frontal lobe.

The dentate nucleus is the largest and most lateral of the CN. It receives inhibitory input from the Purkinje neurons of the large lateral hemispheres and excitatory input from the collaterals of the climbing fibres and mossy fibres projecting to the lateral hemispheres that have their origin in the inferior olive and basilar pontine nuclei, respectively. The neurons of the dentate nucleus project to the red nucleus and the ventrolateral nucleus of the thalamus, which relays the information to the motor cortices of the frontal lobe.

Cerebellar Peduncles

The cerebellum connects to the midbrain, pons, and medulla via three peduncles: the superior, middle, and inferior peduncles, respectively [7, 11]. The superior cerebellar peduncle consists primarily of efferent fibres from the dentate and interposed nuclei projecting to the contralateral red nucleus and ventral lateral nucleus of the thalamus. The cerebellar efferents of the spinocerebellum that project to nuclei of the reticular formation also pass through this peduncle. The cerebellar afferents contained within this peduncle are primarily the fibres of the ventral spinocerebellar tract that project as mossy fibres to the granular layer of the spinocerebellum and send collateral branches to the interposed nuclei.

The middle cerebellar peduncle is a massive bundle of afferent fibres connecting nuclei in the basilar pons to the contralateral cerebellar cortex. These fibres project as the mossy fibres to the granular layer of the large lateral hemispheres and send collateral branches to the dentate nucleus.

The inferior cerebellar peduncle contains fibres connecting the cerebellum to the medulla and consists of the restiform body and the juxtarestiform body. The juxtarestiform body primarily consists of the reciprocal connections of the cerebellum and the vestibular nuclei. The afferent fibres within the juxtarestiform body form the mossy fibres projecting to the granular layer of the vestibulocerebellum. The efferent fibres of the juxtarestiform body include Purkinje cell axons of the vestibulocerebellum and the projections of the fastigial nucleus to vestibular and reticular nuclei of the brainstem. The restiform body contains fibres projecting from the brainstem and spinal cord to widespread areas of the cerebellum. This includes fibres of the dorsal spinocerebellar tract and cuneocerebellar tract projecting to the spinocerebellar cortex as mossy fibres with collateral projections to the interposed nuclei. In addition, fibres originating in the inferior olivary nucleus projecting to the molecular layer of the cerebellar cortex as climbing fibres (with collateral projections to the dentate nucleus) are also contained within the restiform body. The inferior olivary nucleus receives inputs from spinal, vestibular, cranial, and cortical descending signals. The neurons of the inferior olivary nucleus relay somatosensory and noxious stimuli. A single climbing fibre of the inferior olivary nucleus projects to a few Purkinje cells, while each Purkinje cell makes synaptic connections with only one climbing fibre.

The cerebellar cortex also receives projections from a variety of areas of the brain including the locus coeruleus (noradrenergic fibres), raphe nuclei (serotonergic fibres), mesencephalic tegmentum (dopaminergic fibres), and the hypothalamus (histaminergic fibres) [7, 11]. These inputs to the cerebellum terminate in all three layers of the cerebellar cortex as well as the CN. These projections to the cerebellum are commonly referred to as neuromodulatory cerebellar afferents and are thought to decrease the activity of Purkinje cells. The precise distribution and development of these afferent projections to the cerebellum is not well understood. Further research is required to better understand their role in cerebellar function.

Cerebellar Function

The function of the cerebellum can be broadly divided into three categories as set by the three functional divisions described above.

The vestibulocerebellum consists of the midline nodulus and the bilateral flocculе [7, 11]. The mossy fibres projecting to the cortex originate in the vestibular ganglion of the vestibular apparatus and the vestibular nuclei of the brainstem. The Purkinje cells of the cortex send inhibitory projections to the fastigial nucleus as well as the ipsilateral vestibular nuclei. The fastigial nucleus, which serves as the principal cerebellar nucleus of the vestibulocerebellum, sends excitatory bilateral projections to the vestibular nuclei. These connections to the vestibular nuclei pass through the inferior cerebellar peduncle. These projections play an important role coordinating the vestibular ocular reflex via the ascending vestibular nuclei projections contained within the medial longitudinal fasciculus to control eye movement in response to vestibular feedback. The vestibular nuclei also send fibres descending the spinal cord as the vestibulospinal tract. These fibres play a critical role in maintaining balance through activation of the anti-gravity muscles of the lower body. The fastigial nucleus also sends ascending projections via the superior cerebellar peduncle to the ventrolateral nucleus of the contralateral thalamus. This information is subsequently relayed to the corticospinal neurons of the anterior corticospinal tract (medial motor system) involved in maintaining posture and balance through activation of the axial musculature. Lesions of the vestibulocerebellum are often characterized by nystagmus and vertigo, resulting from dysregulation of the connections between vestibular nuclei and brainstem nuclei regulating eye movement. Lesions of the fastigial nucleus are also commonly associated a wide-based gait as a result of instability or ataxia of the axial musculature.

The spinocerebellum consists of the midline vermis and paravermal areas of the cerebellum [8]. The mossy fibres projecting to the cortex are largely the fibres of the spinocerebellar and cuneocerebellar tracts. To a lesser extent, the spinocerebellum also receives input from reticular, vestibular, and pontine nuclei. Although the interposed nuclei receive the bulk of the collateral fibres derived from the ascending inputs into the spinocerebellar cortex, the fastigial nucleus also receives some these collaterals. Similarly, the Purkinje cell axons of the paravermal areas of the spinocerebellar cortex project primarily to the interposed nuclei while the vermal areas project to the fastigial nucleus. The fibres projecting from the interposed nuclei exit the cerebellum via the superior cerebellar peduncle. The majority of these fibres project to the ventral lateral nucleus of the contralateral thalamus, and this information is relayed to supplementary motor, pre-motor, and primary motor cortex involved in regulating the limb musculature. The descending projections of these cortical areas will primarily form the lateral corticospinal tract (i.e., the lateral motor system). The interposed nuclei also send projections to the red nucleus and the reticular nuclei to effect changes in the descending rubrospinal and reticulospinal fibres involved in regulating the activity of the spinal cord motor neurons projecting to the upper and lower limbs. The vermal areas of the spinocerebellum that

project to the fastigial nucleus are primarily involved in regulating axial musculature. As described above, the fastigial nucleus projects to the reticular and vestibular nuclei of the brainstem and the ventrolateral nucleus of the thalamus. Lesions of the vermal portion of the spinocerebellum are characterized by axial muscle instability, while lesions of the paravermal portions of the spinocerebellum produce ataxia affecting the upper and lower limbs.

The pontocerebellum consists of the lateral hemispheres of the cerebellum and constitutes the largest of the three functional components of the cerebellum. The mossy fibres projecting to the pontocerebellar cortex are almost entirely crossing pontocerebellar fibres. These fibres originate in the pontine nuclei of the contralateral basilar pons and enter the cerebellum via the middle cerebellar peduncle. These fibres send collaterals exclusively to the dentate nucleus, which also receives input from the Purkinje fibres of the pontocerebellum. The fibres of the dentate nucleus exit the cerebellum through the superior cerebellar peduncle. These fibres cross the midline within the tegmentum of the caudal midbrain and continue rostrally where some fibres enter into the red nucleus. The neurons of the red nucleus project to the inferior olivary nucleus of the medulla, which projects back to the pontocerebellum and dentate nucleus forming a feedback loop to the cerebellum. The majority of the fibres originating from the dentate nucleus continue past the red nucleus to the thalamus. The fibres terminate on neurons in the ventrolateral nucleus and to a lesser extent in the ventroanterior nucleus of the thalamus. The thalamic neurons contacted by the neurons of the dentate nucleus project rostrally to a large portion of the motor cortices, with an emphasis on the primary motor cortex. The descending neurons from the primary motor cortex form a large component of the lateral motor system. These projections play a critical role in coordinating the muscle activation required for performing fine motor skills of the distal extremities, particularly of the upper limb. The cortical areas regulated by the thalamic relays of the dentate nucleus also play an important role in the planning of motor activity. Lesions of the pontocerebellum are characterized by a decreased ability to control the distance, velocity, and power of movement performed by the extremities. Lesions of the pontocerebellum are commonly characterized by intention tremor and difficulty in performing rapid alternating movements of the hand (e.g., pronation and supination). The latter deficit is referred to as dysdiadochokinesia. These deficits underscore the importance of the pontocerebellum in regulating fine motor skills.

Blood Supply of the Cerebellum

The cerebellum is supplied with arterial blood via three cerebellar arteries: the posterior inferior cerebellar artery (PICA), the anterior inferior cerebellar artery (AICA), and the superior cerebellar artery (SCA) [12]. These arteries are derived from the vertebral–basilar arterial system that supplies the posterior circulation of the brain. The bilateral vertebral arteries pass through the foramen magnum and shortly after entering the cranium, the PICA branches off the vertebral artery. The

PICA supplies the cortex of the posterior portion of the inferior cerebellum and the inferior portion of the underlying white matter. It also supplies the fibres of the inferior cerebellar peduncle. The vertebral arteries fuse in the midline, near the junction of the pons, and the medulla, to form the basilar artery, and the AICA branches off the basilar artery immediately anterior to this junction. The AICA supplies the cortex of the anterior portion of the inferior cerebellum and the underlying white matter. Distal branches of the AICA may extend into the lateral portion of the dentate nucleus. The AICA also supplies the posterior part of the middle cerebellar peduncle, while circumferential branches of the basilar artery supply the anterior portion of the middle cerebellar peduncle. The most lateral edge of the inferior surface of the cerebellum is generally the watershed area of the PICA and the AICA.

The SCA attaches to the basilar artery immediately posterior to the bifurcation of the basilar artery into the paired posterior cerebral arteries. The SCA supplies the superior surface of the cerebellum and the bulk of the white matter of the cerebellum. It also supplies the CN except for the lateral portion of the dentate nucleus that may be supplied by the AICA. The SCA also supplies the superior cerebellar peduncle together with branches of the posterior cerebral artery.

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Cellular and Genetic Programs Underlying Cerebellum Development

Alexandra L. Joyner, Ryan Willett, and Andrew Lawton

Abstract The cerebellum is a late developing structure compared to the rest of the central nervous system (CNS) and houses more cells than the entire rest of the brain in a complex set of folds. To accommodate production of the large number of cells, the cerebellum has not only a ventricular progenitor zone that produces all the glia and inhibitory neurons but also a unique progenitor zone, the rhombic lip, dedicated to excitatory neuron production. In this chapter we discuss how the inhibitory Purkinje cells, which integrate the incoming information and moderate the output neurons of the cerebellar nuclei, play a key role during development in ensuring appropriate production of the other neurons/astrocytes of the cerebellar cortex. Key transcription factors that regulate development of the two progenitor populations and the lineage relationships of the neurons and astrocytes produced by each are described, followed by a discussion of cerebellar foliation.

Keywords Ventricular zone • Rhombic lip • Purkinje cells • Granule cells • Interneurons • Bergmann glia • Astrocytes • Cerebellar nuclei • Neural stem cells • Foliation

Introduction

The cerebellum is the region of the brain that is the latest to complete neurogenesis; in humans cerebellar development continues during the first year of life and in mouse for more than 2 weeks after birth [1–3]. It arises from the dorsal aspect of the most anterior hindbrain called rhombomere1 (Fig. 1a, b). Remarkably, the volume of the human cerebellum increases ~10× between 20 and 40 weeks of gestation, with the surface area increasing much more due to the formation of folia and lobules [6–8]. The mouse cerebellum undergoes maximum growth and foliation after birth (Fig. 1a–d). Given the late development of the CB compared to other brain regions,

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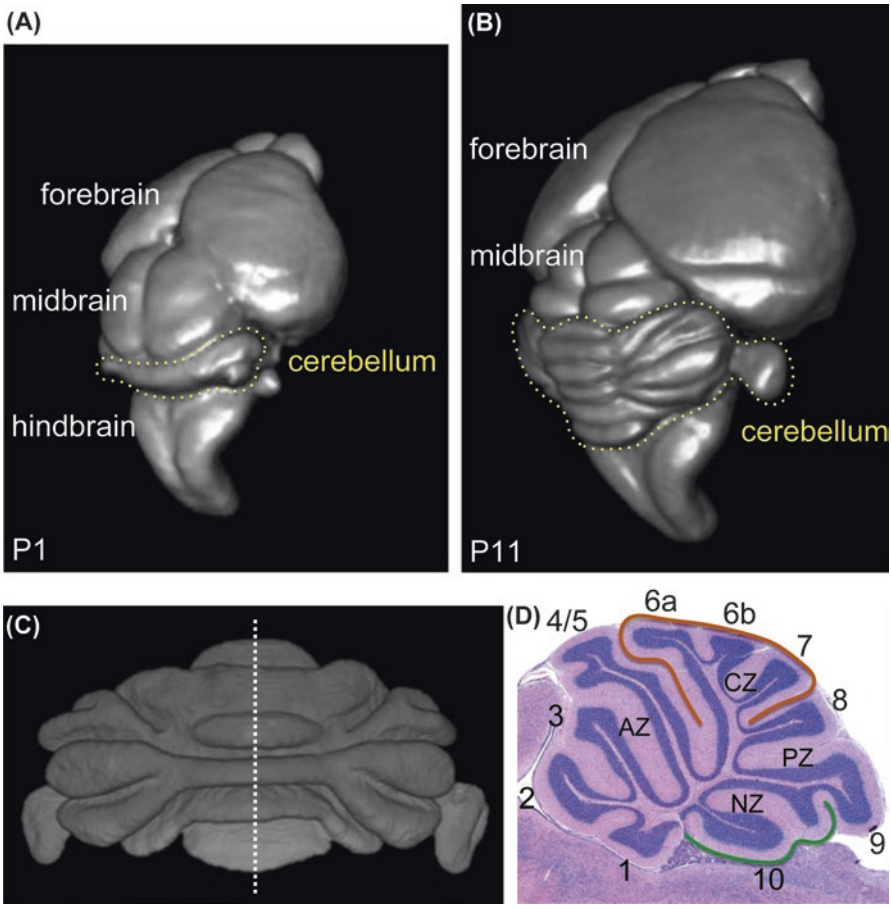

Fig. 1 The cerebellum forms in the dorsal anterior hindbrain and has its major growth and foliation after birth. (**a–c)** microMRI images illustrating mouse postnatal cerebellum development (outlined in *yellow*) (based on [4]), and (**c)** highlighting distinct foliation patterns in the medial vermis and lateral hemispheres [5]. (**d)** Hematoxylin and eosin (H&E) midline section (*dotted line* in **c**) of adult cerebellum. 1–10, lobules, *AZ* anterior zone, *CZ* central zone (outlined in *red*), *PZ* posterior zone, *NZ* nodular zone (*green*)

the cerebellum is particularly sensitive to environmental and clinical factors that impact on growth (or cause injury) around birth [9]. A better understanding of the factors that regulate progenitor cell expansion, production of neurons and glia, and their compartmentalization during foliation should pave the way for developing therapeutic approaches to stimulate endogenous progenitors to replenish cells lost due to injury.

The developing cerebellum is unique among the brain regions as it has two zones that house neural stem and progenitor cells (Fig. 2a). Whereas in the rest of the central nervous system the ventricular zone (VZ) gives rise to all the neurons and

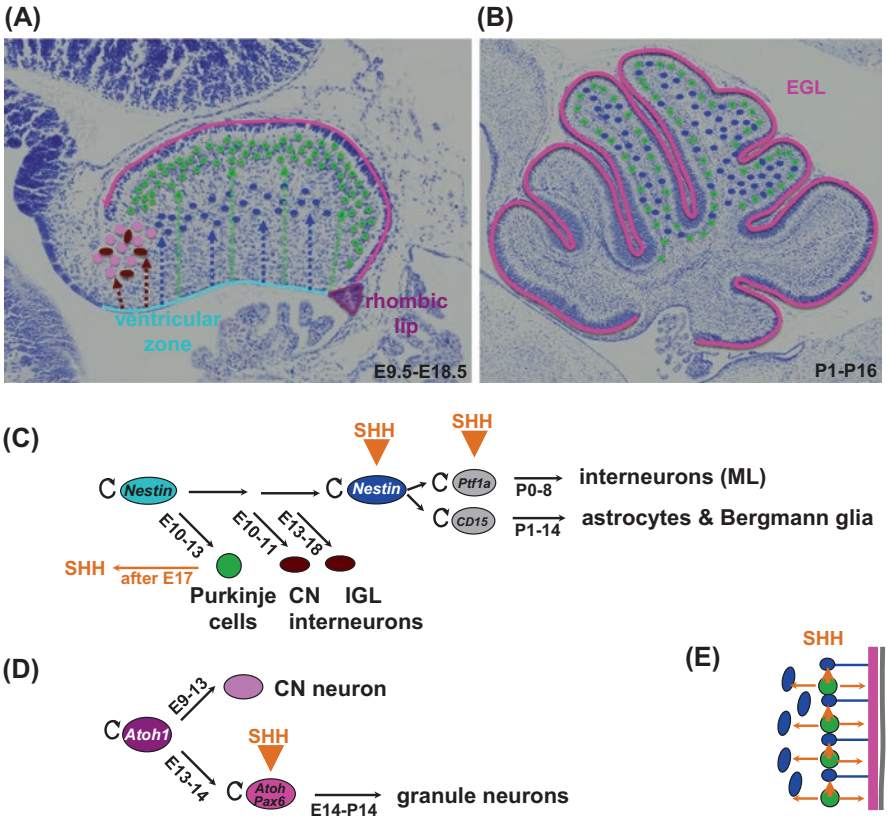

Fig. 2 Two progenitor zones produce all the neurons and the astroglia of the cerebellum at particular time points. (**a**) Midline eosin stained sagittal section of E16.5 cerebellum with ventricular zone (*turquoise*) and rhombic lip (*pink*) indicated and the cells that arise from the zones color coded as in **c** and **d**. (**b**) Midline sagittal section of P3 cerebellum showing EGL (*pink*), Purkinje cells (*green*), and Nestin-expressing progenitors. (**c**) The ventricular zone lineage is shown. (**d**) The rhombic lip lineage is shown. (**e**) SHH (*orange*) is expressed by Purkinje cells and signals to all progenitors in the postnatal cerebellum, also indicated in **c** and **d**

glia, the VZ of the cerebellum is dedicated to making only inhibitory neurons (Purkinje cells and interneurons), as well as astrocyte-like glia (astrocytes and Bergmann glia referred to as astroglia) [10]. Interestingly, most of the interneurons and astroglia are generated from intermediate progenitors that leave the VZ and proliferate after birth in the cerebellar cortex [11–14] (Fig. 2b, c). The second cerebellar progenitor zone is called the rhombic lip and generates the excitatory neurons of the cerebellum, primarily the granule cells, and projection neurons of the cerebellar nuclei [15–17] (Fig. 2a, d). Like the astroglia and interneurons, the granule cells are generated from a secondary progenitor pool made up of granule cell precursors (GCPs) that are housed in the external granule cell layer (EGL) that covers the surface of the cerebellum during development and generates granule cells that migrate

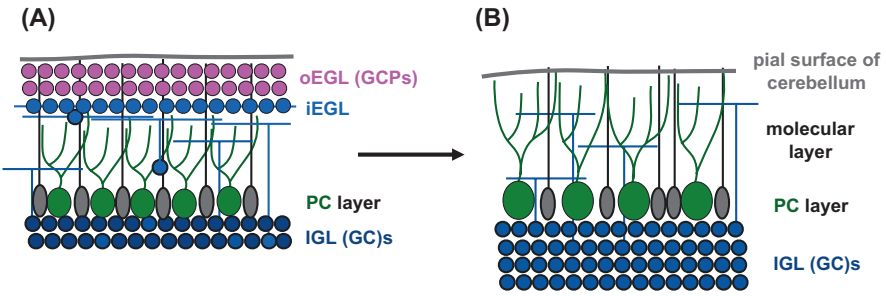

Fig. 3 Schematic drawing showing granule cell development. (**a**) During development (E15.5-P14), the cerebellum is covered with granule cells organized in a layer called the external granule cell layer (*EGL*) which is divided into an outer layer (*oEGL*) of dividing progenitors (*GCPs*) and inner layer (*iEGL*) of postmitotic granule cells (*GCs*) that extend parallel fibers (axons shown as horizontal *blue lines*). GCs migrate down the fibers (*black lines*) of Bergman glia (*grey* cell body) past the Purkinje cells (PCs, *green*) to form the inner granule cell layer (*IGL*). **(b)** Newly formed parallel fibers stack on top of older ones to form the molecular layer that also has interneurons (not shown), but the cell bodies of GCs randomly mix in the IGL. PCs express SHH, which is required for GCP proliferation

inward to form the internal granule cell layer (IGL) (Figs. 2a, b and 3). In humans, the EGL reaches a maximum volume after birth [2]. It is tempting to speculate that a dedicated transient amplifying progenitor pool evolved for the granule cells, because the granule cells comprise a majority of the neurons in the brain and thus require massive expansion of progenitor numbers during development. Curiously, the source of most oligodendrocytes for the cerebellum appears to be the VZ outside the cerebellum, likely the midbrain and/or ventral rhombomere 1 [18–20].

In this chapter, we use mouse as a model system to describe development and foliation of the cerebellum (Fig. 1) and the generation of the various neurons and astroglia of the cerebellum since precise knowledge of the VZ and RL lineages has been obtained with genetic fate mapping studies. Cumulative fate mapping with a site-specific recombinase such as Cre labels all cells that ever expressed Cre, and if the gene is specific to one progenitor pool, then all the cell types generated from the pool can be determined [21] (Fig. 4). The temporal sequence of cell-type generation is determined by genetic inducible fate mapping (GIFM). This method only labels cells expressing Cre during a particular ~24 h period [22]. Furthermore, using GIFM, the initial marked population can be precisely determined, as well as the descendants of the population at any later developmental stage or in the adult. Using promoters specific to each stem/progenitor population, detailed knowledge of the cerebellar lineages has thus been uncovered.

In this chapter we define the lineage relationships of each stem/progenitor pool, the temporal pattern of cell-type generation, and some of the proteins that regulate progenitor cell number expansion and differentiation. We include a discussion of how the numbers of each neuron/astroglial type in the cortex might be scaled to attain the correct relative proportions of different cell types and the possible contributions of the progenitor pools for replenishment of cells after an injury at

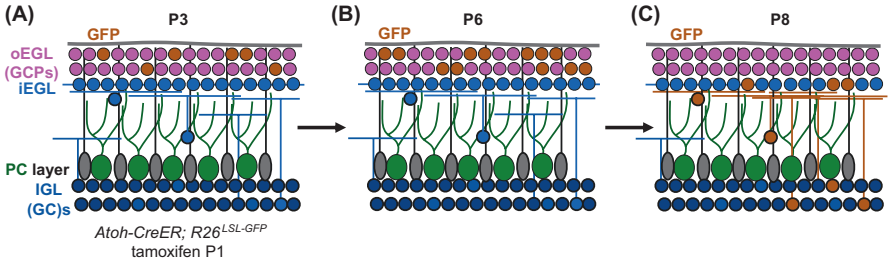

Fig. 4 Schematic illustrating genetic inducible fate mapping (GIFM). An *Atoh1-CreER* transgene is expressed only in granule cell precursors (*pink*, *GCPs*) in the outer external granule cell layer (*oEGL*). A reporter allele, $R26^{LSL\text{-}Gfp}$, expresses GFP in cells that have active Cre. Tamoxifen is injected into *Atoh1-CreER,* $R26^{LSL\text{-}Gfp}$ mice at P1, and it binds CreER and allows it to move from the cytoplasm to the nucleus and induce recombination of loxP sites in the $R26^{LSL\text{-}Gfp}$ allele (*LSL* = loxP-stop of transcription sequence-loxP), which allows GFP expression. A small number of GCPs are initially labeled with GFP (*brown*) (**a**) and then expand in number (**b**) and then differentiate (**c**). All cells in a clone differentiate at the same time; a clone is shown in **c**. Colors and labels are as described in Fig. 3

birth. This is especially relevant to premature births, since the cerebellum is particularly vulnerable to clinical and environmental factors around birth because much of its growth occurs in the third trimester and continues after birth. We end with a description of how the complex three-dimensional folded structure of the cerebellum develops in mouse and discuss how particular efferent neural circuits are enriched in specific subsets of lobules and the possible implications of this spatial division of functions for evolution of new cerebellar functions.

Early Patterning of the Neural Tube and Specification of the Cerebellar Territory

The cerebellar anlage is specified in the dorsal aspect of the anterior hindbrain called rhombomere 1 around embryonic day 9 (E9) in mouse [23–26]. Chick transplantation studies around two decades ago demonstrated that the boundary between the midbrain and hindbrain (referred to as the isthmus) is an organizing center that initiates development of r1 and the midbrain (reviewed in [27–29]). Dorsally an epithelial structure (isthmus) can be seen at E18.5 in mouse that links the cerebellum to the tectum (Fig. 5). The key isthmic organizer gene is *Fgf8* (fibroblast growth factor 8), as it is expressed in the isthmus (E8.5–12.5), is required to induce formation of the anlage of the midbrain and r1 [30], is sufficient to induce and pattern the midbrain and rhombomere 1 [31, 32], and is necessary up until E12 for cerebellum development [30, 33]. The secreted factor WNT1 is also expressed near the isthmus and is required for development of the midbrain and cerebellum [34, 35]. The molecular interactions of FGF8 with the transcription factor OTX2, required in the midbrain,

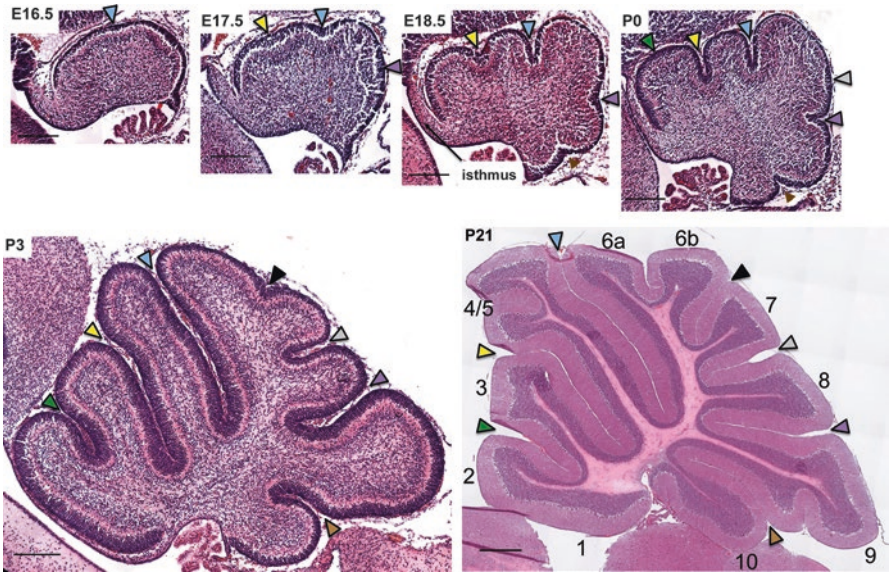

Fig. 5 Stereotypical formation of fissures during mouse cerebellum development. Midsagittal H&E sections of the cerebellum at the indicated stages. The same fissures are indicated by colored *arrowheads*. The lobules are numbers at P21. *Line* indicates 200 μm for E18.5-P3 and 500 μm for P21

and GBX2, required in the hindbrain, have been reviewed extensively, and we refer you to a detailed recent review by Martinez [28]. The dorsal-ventral axis of r1 and the midbrain is determined primarily by the morphogen sonic hedgehog (SHH), expressed by the ventral midline or floor plate [36–38]. The engrailed homeobox transcription factors (EN1/EN2) are key target patterning genes of both FGF8 and WNT signaling, with *En1* being required for the initial formation (specification) of most of the midbrain and r1 and the two genes then involved in regulating growth and foliation of the cerebellum [39, 40]. Double-mutant experiments, including conditional removal of the genes in particular lineages, have revealed overlapping and unique roles of *En1* and *En2* after the cerebellar territory is established [41–43].

Ventricular Zone Lineage

Cumulative genetic fate mapping using a line of mice in which *Cre* was inserted by gene targeting into the *Ptf1a* gene (knockin) (*Ptf1a*^{Cre}), demonstrated that only inhibitory neurons are generated from the VZ, as well as astrocytes and Bergmann glia [10] (Fig. 2c). Traditional ^{3}H-thymidine or BrdU birth dating experiments and GIFM using *Ascl1*^{CreER} revealed that Purkinje cells and interneurons of the cerebellar nuclei (CN) are the first neurons to be born during E10–13, with the interneurons being born over a shorter period [1, 12] (Fig. 2d). Interneurons are then born in an

inside (IGL) to outside (outer molecular layer) spatial progression [12, 14]. During the production of Purkinje cells, the GLI3 repressor side of the SHH pathway may play a role in proper production of ventricular zone-derived cells [37, 44]. Astrocytes and oligodendrocytes are primarily born after birth. A chick-quail chimera analysis traced the main source of oligodendrocytes to the VZ of the midbrain [19]. An earlier study in mouse also using transplantation provided evidence that the source for oligodendrocytes in the mouse cerebellum is also outside the structure and showed that oligodendrocyte precursors populate the cerebellum around E15.5 and then expand in number [20]. A recent fate mapping study argues mouse oligodendrocytes are derived from the hindbrain [18]. Curiously, a small population of Bergmann glia is born at around E13.5 [12], but most are born after birth during the major growth phase of the cerebellar cortex [11, 12, 45]. In addition, the interneurons that settle in the IGL and CN are the main interneurons derived directly from the VZ.

Interestingly, Purkinje cells have distinct settling patterns under the surface of the cerebellar cortex, depending on the day they are born, with successive waves of Purkinje cells forming different wide stripes along the anterior-posterior axis [12, 46]. Purkinje cells throughout the cerebellum initially settle into an aggregate of cells called the Purkinje plate at E14.5 before migrating outward to settle into a multilayered Purkinje cell layer (PCL) by E18.5 under the cerebellar surface. As expansion of the cerebellum continues through the postnatal growth phase, Purkinje cells resolve into a monolayer by approximately postnatal day 5 (P5) [12, 47]. Purkinje cells in the lobules of the central zone (CZ in Fig. 1d) are the last to form a monolayer, correlating with delayed generation of granule cells in these lobules [48].

The Purkinje cells initially exhibit simple morphology of a leading apical neurite and trailing axon left behind as they migrate to the PCL from the Purkinje plate (fusiform). At around P0 they undergo a sequence of cell shape changes; first their apical neurite collapses and the cells take on a stellate morphology with numerous short perisomatic neurites (~P6), and then they evolve a distinct bipolar morphology with a highly elaborated dendritic configuration that is flattened in an ramified espaliered fashion within the sagittal plane (P8 onward, [49]). The Purkinje cells of the central zone are the last to differentiate.

A medial-lateral corticonuclear topographic projection map of Purkinje cell axons to the cerebellar nuclei can be seen as early as E15.5 in mice [50], and electrophysiological recordings can be made early postnatally. While the vast majority of Purkinje cell axons project into the cerebellar nuclei, Purkinje cells of the flocculus, paraflocculus, and the nodulus of the vermis (lobule 10) instead route into the vestibular nucleus of the hindbrain.

Postnatal Cerebellar Cortex Progenitor Populations and Lineages

Ventricular zone-derived progenitors are present in the postnatal cerebellar cortex and proliferate and give rise to interneurons in the molecular layer for about a week after birth in mouse (Fig. 2b, c). These progenitors also give rise to astrocytes and

additional Bergmann glia for over a week after birth [11–14]. Elegant genetic fate mapping studies combined with marker analysis were used to address the location and lineage relationships of stem/progenitors in the cerebellar cortex [11, 45]. Using several *CreER* lines (*GLI1*^{CreER}, *Tnc*^{CreER}, *Ptf1a*^{CreER} knockin alleles) to mark Nestin-expressing stem/progenitor cells, and proteins that mark interneurons (PAX2) or astrocytes (GFAP), it was found that *Tnc*- and *Cd133*-expressing multipotent progenitors give rise to both a unipotent *Ptf1a*-expressing progenitor that expands the interneuron population during the first week after birth and to a *Tnc*- and *Cd15*-expressing progenitor dedicated to the astroglial lineage that likely gives rise to both astrocytes and Bergmann glia [11]. PAX2^{+} immature interneurons are generated in an inside-to-outside manner (basket and then stellate interneurons) in the molecular layer and then mature during the first few weeks after birth. A recent study addressed the location of the multipotent and unipotent progenitors using a *Glast*^{CreER} allele [45]. Tamoxifen was administered to the surface of the cerebellum to label only astroglial cells in the Purkinje cell layer that had a radial process extending to the surface. Interestingly, they demonstrated that *Glast*^{CreER} cells in the Purkinje cell layer generate new Bergmann glia and astrocytes, whereas progenitors in the white matter generate astrocytes and interneurons based on a clonal analysis. Finally, Nestin-expressing progenitors situated along the inner edge of the EGL have been proposed to produce GCPs [51], but it seems possible they normally give rise to interneurons in the white matter.

In vitro stem cell assays support the in vivo genetic fate mapping demonstration of multipotent stem/progenitor cells in the early postnatal cerebellar cortex. Stem cells isolated from the P3–7 cerebellum by FACS based on expression of CD133^{+} and the absence of lineage markers (PSA-NCAM, TAPA-1, and O4) or cells with a low level of *Tnc*$^{YFP\text{-}CreER}$ that also express *Cd133* and *Gli1* can form multipotent clonal neurospheres in culture that can differentiate into the expected interneurons and astrocytes, as well as granule cells and oligodendrocytes [11, 52]. Moreover, when transplanted into a P3 cerebellum, the cells form rare Purkinje cells, as well as many interneurons, astrocytes, and oligodendrocytes [52]. Since almost all CD133^{+} cells express *Tnc*$^{YFP\text{-}CreER}$ (and thus SOX2 and Nestin) [11], these studies indicate that cerebellar stem cells have a greater differentiation capacity (plasticity) in vitro than is seen during normal development. In another study, cells taken from the cerebellum of E14.5, P0, or adult mice and depleted of GCPs (ATOH1^{-}) also formed multipotent neurospheres with a similar differentiation capacity to CD133^{+} stem cells in culture and after transplantation [53]. Thus, rare stem cells remain in the adult cerebellum that can form most neuron types and glia when presented with the appropriate environment. These results raise the possibility that rare quiescent stem cells in the early postnatal or adult could be mobilized to replace neurons or glia after an injury if the necessary inducing factors can be identified.

Purkinje cells play a key role in growth of the cerebellum, as they express the mitogen sonic hedgehog (SHH), [13, 54, 55] which signals to both GCPs and white matter stem/progenitor cells [11] (Fig. 2c–e). SHH signaling in GCPs is required for their proliferation and viability after E16 [54, 56, 57]. Furthermore, deletion of *Shh* in Purkinje cells or ablation of HH signaling in white matter stem cells reduces expan-

sion of the pool of *Tnc*^{CreER}-labeled white matter stem/progenitor cells and production of interneurons and astroglia [11]. In addition, application of SHH to cerebellar slice cultures stimulates interneuron production [58]. Purkinje cells can coordinate growth of all cell types produced in the cerebellar cortex except, possibly oligodendrocytes, via SHH secretion (reviewed in [59]). How SHH is delivered from Purkinje cells to the outer EGL and white matter progenitors and whether there are other sources of HH ligands that regulate cerebellar neurogenesis remain open questions.

The bHLH transcription factor PTF1a is key to VZ cells, as in its absence all cerebellar inhibitory neurons are lost and astrocytes are depleted [10, 60, 61]. Interestingly some VZ-derived mutant cells are transformed into rhombic lip-derivative neurons and cell types normally generated from the VZ ventral to the cerebellum. Furthermore, PTF1a is sufficient to largely specify a generic inhibitory cell phenotype, as ectopic expression of PTF1a in several excitatory neuron progenitors in the nervous system induces a network of inhibitory neuron gene expression and repression of excitatory neuron genes [10, 62]. The related bHLH protein-encoding gene *Ascl1* plays a more limited role in generation of cerebellar interneurons [12]. Curiously, climbing fiber neurons also require *Ptf1a* for their survival, migration, and differentiation from the more posterior hindbrain, and in the absence of *Ptf1a*, some precursors take on a mossy fiber fate [63]. Genes that regulate Bergmann glia generation and function are absolutely critical for cerebellar growth, foliation, and formation of a normal cortical architecture [64].

Rhombic Lip Lineage

The rhombic lip (RL), formed by E9.5 at the posterior rim of the cerebellar anlage where the pial surface contacts the ventricular zone, is the source of all glutamatergic neural subtypes of the cerebellum (Fig. 2a,c). Cells arising from the RL spread anteriorly across the surface of the cerebellar anlage and sequentially produce two cell populations: postmitotic cerebellar nuclei (CN) and then proliferating granule cell precursors. Lineage tracing and birth dating studies have shown that the earliest population of cells emerging during E9.5–E12.5 migrate to and accumulate into two clusters of cells bilaterally symmetrically displaced from the midline, known as the nuclear transitory zone [16, 17]. Immature CN cells migrating from the rhombic lip to the nuclear transitory zone are ATOH1^{+}/PAX6^{+}, and as they migrate into the nuclear transitory zone, the proteins are downregulated and CN progenitors sequentially express TBR2, TBR1, and reelin [65].

Cells leaving the rhombic lip from E13.5 onward become cerebellar granule cell precursors (GCP) [16]. These cells remain at the cerebellar surface for the duration of embryonic development and form a dense proliferative layer called the external granule layer (EGL). As development advances, growth of the cerebellar anlage and concomitant EGL expansion subsume the nuclear transitory zone into an interior position where they are reorganized into two bilateral groups of three distinct nuclei in mouse: (from medial to lateral) the fastigial, interpositus, and dentate nucleus. The

three individual nuclei are clearly distinct by birth in mouse, and TBR1 or BRN2 expression marks the fastigial nuclei or the interpositus and dentate nuclei, respectively [65]. The human CN shows a massive expansion of the dentate nucleus compared to mouse, likely due to the vast expansion of hemisphere lobules. Additionally, two separate nuclei are found in human in the place of the mouse interpositus: the human globose and emboliform nuclei. The Purkinje cell axons converging on the CN become myelinated during postnatal gliogenesis. In the mature cerebellum, the CN reside in the confluence of white matter just dorsal to the cerebellar peduncles.

Initiation of SHH expression in Purkinje cells at E17.5 profoundly enhances GCP proliferation and commences the main period of granule cell neurogenesis that drives the major portion of cerebellar growth (Fig. 1a, b). At this time the EGL takes on a bilayer structure; the outer EGL (oEGL) contains the actively proliferating GCPs, and the inner EGL (iEGL) is populated by postmitotic and differentiating GCPs (Fig. 3). The GCPs of the iEGL migrate medial-laterally for approximately a day before they descend along Bergmann glia fibers to create the inner granule layer (IGL). As they descend, the incipient granule cells (GCs) leave a trailing apical neurite in the molecular layer, which bifurcates into a parallel fiber that extends medial-laterally and synapses onto Purkinje cells.

The bHLH protein ATOH1 is required for generation of GCPs and for most cerebellar nuclei projection neurons [17, 66]. One function of ATOH1 is to induce *Gli2* expression, and thus to enhance SHH signaling in GCPs [67], and likely regulate many other genes required for granule cell proliferation (e.g., *MycN* and cyclin D1) and differentiation [68]. There appears to be an antagonistic relationship between the rhombic lip protein, ATOH1, and the VZ transcription factor PTF1a, as mis-expression of each protein in the complementary progenitor zone leads to inhibition of the other gene [69]. Mossy fiber neurons also require *Atoh1* for their development [17].

Granule Cell Precursor Cell Behaviors

The role of granule cells in cerebellar development and function and the identification of GCPs in the etiology of the tumor medulloblastoma [70] have attracted interest in their proliferative behaviors. Of particular interest is how the expansion of the GCP population drives postnatal cerebellar growth and morphology (foliation). Analysis of GCP clones revealed that granule cell precursors primarily undergo symmetrical divisions to expand the number of the cells in a clone during postnatal development [71–73]. Shortly before the clones differentiate, the GCPs within a clone undergo an added burst of proliferation before they differentiate over a small temporal window. A single GCP at the E17.5 stage produces an average of 250 granule cells, requiring at least eight cell divisions. Despite the degree of synchrony in proliferative behaviors and differentiation timing of these GCP clones, individual cells within the clones over time exhibit poor synchrony of cell cycle phases [72].

Clonal studies have also provided insight into how the complex form of the cerebellum is shaped. During postnatal development, the cerebellum expands to a far

greater extent along the anterior-posterior axis than in the medial-lateral axis, due in part to orientation bias of the GCPs to divide in the anterior-posterior axis along with tangential migration within the EGL [72]. Conversely, as GCPs differentiate into nascent GCs and descend into the IGL, they favor a medial-lateral spread within the growing lobule. Lineage birth dating studies capable of labeling these parallel fibers have shown that parallel fibers are laid down in an inside-to-outside fashion with the earliest born granule cells innervating the deep molecular layer and the late born GCs innervating the outmost extent of the molecular layer [48, 73] (Figs. 3 and 4). In contrast to the ordered laminar arrangement of parallel fibers, the cell bodies of new GCs settle at random depths in the underlying IGL. The base of each fissure that separates the cerebellum into lobules acts as a boundary to the movement of GCPs [72]. Thus, after fissure formation GCPs are maintained in the nascent lobule. This intriguing finding suggests that lobules may not simply be anatomical units, but could also have functional uniqueness and act as separate developmental units.

Regional differences appear in the cerebellum with respect to granule cell proliferation and differentiation. Granule cell production in the anterior (lobules 1–5) and posterior (lobules 8–10) cerebellum predominates over the central region (lobules 6/7) in the perinatal period, but this delayed growth in the central zone is compensated for by the perdurance of a thicker EGL in lobules 6/7 around P14, whereas the EGL is exhausted in all other cerebellar regions [48]. Taking these observations together with a delay in onset of SHH expression in lobules 6/7 and that the intercrural and declival fissures are the latest vermis fissures to form, a picture emerges that the central zone encompassing lobules 6/7 has a general developmental delay that continues for days after cessation of development in the rest of the cerebellum. It will be interesting to determine the dynamics of regional growth in the vermis of the human cerebellum, as well as in the hemispheres.

Development of Cerebellar Afferents

Climbing fibers from the inferior olive innervate the cerebellar anlage as a fasciculated axon bundle beginning at E15.5–E16.5 in mice, and by late E16.5 the first synapses with Purkinje cells are observed [74–78]. By birth, these axons defasciculate and innervate the developing Purkinje cell multilayer, with each Purkinje cell receiving multiple climbing fiber inputs [79]. The supernumerary climbing fiber inputs are eliminated in an activity-dependent fashion between the second and third postnatal week so that each adult Purkinje cell is innervated by a single climbing fiber axon [80–82]. Mossy fibers arrive in the cerebellar anlage between E13.5 and E15.5 [83] and form transient contacts with Purkinje cells by birth. Within the first postnatal week, the mossy fibers establish cell-cell contacts with synaptic ultrastructural features, but in the second postnatal week, they withdraw to refine their synaptic connections with their proper GC and Golgi cell targets in the internal granule layer [84].

Development of Cerebellar Foliation and Relationship to Afferent and Efferent Circuitry

During development the cerebellum increases in size and undergoes a dramatic progressive foliation transforming the smooth outer surface into a highly foliated collection of lobules separated by fissures (Figs. 1 and 5) [85]. In the human cerebellum, there are additional shallower folia along the surface of the lobules, and they form at an early stage of the fetal foliation process. Foliation creates dramatically more surface area in the cerebellum along the anterior-posterior axis, maximizing the number of cells in the layered cortex and thus the quantity of functional circuits that the cerebellum can host in the spatial constraints of the posterior skull. Foliation also correlates with spatial separation of distinct functional regions within the cerebellum. Afferents to the cerebellum from the spinal cord and brain target particular locations within the medial-lateral axis of the cerebellum. Furthermore they project to particular lobules [86]. For example, the spinocerebellar circuit projects only to the anterior and posterior zones of the vermis. Within the lobules, some circuits target regions that correspond to the longitudinal cerebellar zones (stripes) defined by different gene expression [86, 87]. There is also a spatial relationship between Purkinje cells and the cerebellar nuclei they project to, generally medial to lateral, but it seems likely there is also an anterior-posterior code. Thus, efferent functions have spatial domains.

The murine foliation pattern is highly consistent across individuals and has minor strain-specific variation [88]. The pattern of foliation varies depending on the medial-lateral position in the cerebellum (Fig. 1c), and fissures form in a specific sequence (Fig. 5). In mouse, as in all mammals, the medial cerebellum, or vermis, has ten primary lobules [86, 87]. The lateral hemispheres have their own distinct pattern as do the most lateral paraflocculi and flocculi.

Foliation begins during the last embryonic days, around E16 to E17, and the last fissure begins to form by ~P5 (Fig. 5). The first indication of foliation at E16.5–17.5 is a regional inward thickening of the EGL, which will correspond to the base of the newly forming fissure. Following this thickening of the EGL, the outer surface of the cerebellum indents (Figs. 5 and 6). Around this time GCPs within the base of the forming fissure become elongated, and the local Bergmann glia direct their fibers to the center of the indention (Fig. 6b) [89]. The intervening regions between the fissure bases expand outward. By following the foliation process through to completion, it can be seen that the fissure bases hold their relative spatial positions and thus are called anchoring centers, as the lobules expand to their final size [4, 89].

As discussed previously, the proliferation of GCPs in the EGL and the resulting growth of the cerebellum are dependent on SHH supplied by the underlying Purkinje cells [54, 56, 57]. Reducing the level of SHH signaling reduces the overall growth of the cerebellum and concomitantly reduces the degree of foliation. The EGL becomes thinner and the first appearance of anchoring centers is delayed. Additionally, foliation is precociously halted. However, the fissures that do form correspond to the earliest fissures suggesting that while SHH provides the growth that is necessary for foliation to proceed, it does not control the pattern of foliation.

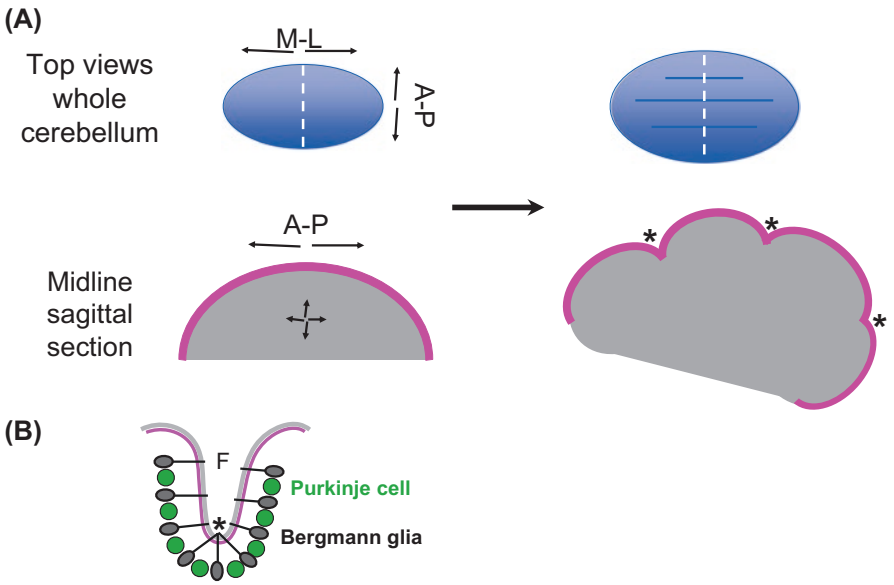

Fig. 6 Model of cerebellum foliation based on differential expansion of layers. (**a**) As the outer layer, the EGL (*pink*) expands more rapidly than the inner mass of the cortex (*grey*), the EGL buckles creating anchoring centers (*). White *dotted lines* in top views indicate where sagittal sections are positioned. Fissure placement is proposed to be directed by the differential expansion of the layers and the shape (*ovoid*) of the initial cerebellar anlagen. (**b**) The Bergmann glial fibers (*black*) connect the outer surface (*thick grey line*) to the inner buckling Purkinje cell (*green*) layer that contains the cell bodies of the Bergmann glia (*dark grey*), and a fissure (*F*) forms above each anchoring center as the cerebellum continues to expand. *A-P* anterior-posterior, *M-L* medial-lateral

When the level of SHH is increased beyond wild-type levels, the cerebellum is larger and has an extra fissure [57]. Intriguingly, this extra fissure is placed in a conserved position similar to where the rat has an additional fissure. Consistent with the requirement for HH signaling in GCP proliferation, induction of activating HH signaling mutations specifically in the GCP lineage results in the SHH subgroup of medulloblastoma [70, 90, 91].

The proliferation of GCPs is temporally and spatially regulated within the cerebellum. Maximum proliferation in the lobules of the central zone (6–7) is delayed and maintained longer relative to the other cerebellar zones. This difference is attenuated in the cerebellum of $En1^{+/-};En2^{-/-}$ mutants that have an abnormal foliation pattern such that proliferation in the anterior, posterior, and nodular zones is more similar to the central zone [48]. Because granule cells do not disperse across fissure boundaries, this creates self-contained lobules; this allows any lobule-specific granule cell behavior to fine-tune the shape of the lobules [72].

Blocking the generation of Bergmann glial cells has revealed that there are at least two separable stages of anchoring center formation: an inward thickening of the EGL and formation of an indentation on the outer cerebellar surface. The cere-

bellum is covered by the pial surface as well as the end feet of the Bergmann glial processes. In the absence of Bergmann glia, the EGL thickens, but the outer edge of the cerebellum fails to subsequently bend inward. Consequently, fissures fail to appear at the cerebellar surface. Nevertheless, many granule cells are displaced deep into the cerebellum and form a fissure-like mass, possibly at the positions of the initial EGL thickening. As a result, the layers of the cerebellum are not well defined, and the foliation pattern is severely disrupted when Bergmann glial development is disrupted [64, 92, 93].

In addition to acting as a physical bridge between the outer surface of the cerebellum and the Purkinje cell layer, Bergmann glial fibers provide the scaffolding for the radial migration of newly born granule cells from the EGL to the inner granule layer (Fig. 3). Disrupting the development, or orientation, of Bergmann glial fibers thus leads to aberrant GC migration and the ectopic accumulation of GCs in the molecular layer. In some cases this disruption is severe and can distort foliation [94]. Thus, the Bergmann glia play a key role in cerebellar foliation and formation of a normal cytoarchitecture.

Alterations in the timing of anchoring center appearance change the resulting foliation pattern. In *En2* null mutants, the appearance of the anchoring centers for the secondary and prepyramidal fissures surrounding lobule 8 is reversed in developmental time. This results in a lengthening of the prepyramidal fissure and a shortening of the secondary fissure and a corresponding foliation pattern change in the intervening lobule 8 [39, 42]. Interestingly, the initial changes in the EGL and Bergmann glia that signal the formation of an anchoring center appear normally even when the entire anchoring center either forms prematurely or is delayed [89].

Little is known about the tissue-scale mechanical or physical forces that underlie cerebellar foliation. However, the cerebral cortex is also a folded tissue in primates, and many models have been proposed to describe the formation of sulci and gyri during cerebral gyrification. Many of these models are based on a system of differential or constrained growth of a tissue bilayer. Differential growth rates between connected layers can lead to tissue buckling and subsequent surface folding. These models take into consideration that the pattern of foliation can be shaped by adjusting the starting size of the tissue, the difference in the growth rates of the layers, and the mechanical properties of the layers [95–100]. It is interesting to speculate whether similar forces could be responsible for the initial appearance of anchoring centers in the cerebellum (Fig. 6a). Like the cerebral cortex, the cerebellar cortex can be considered as divided into multiple layers. One model of cerebellar folding used a tri-layer model of differential growth [101]. In this model the EGL and the IGL were considered separated by a “soft” Purkinje cell layer. This three-layer system when modeled to have a higher outer growth rate allowed for surface wrinkling even if the outer and inner layers had similar measures of stiffness. These models suggest that the comparative rates of growth of the outer and inner cerebellum are important to foliation, as is the initial shape of the cerebellar anlage.

It is exciting to speculate about the evolution and functionality of the compartmentalized lobule structure and the spatial segregation of afferent project fields to particular lobules and zones. The cerebellum is involved in diverse roles including

cognition and social behaviors. The cerebellar hemispheres have undergone tremendous expansion in humans, and they house the majority of long-range circuits that involve the neocortex. It is possible that as the neocortex expanded and became folded into gyri and sulci, there was similar spatial segregation of neuronal circuits into particular neocortex folds. This would be one way for developmental programs to be divided into subunits that could have separate regulatory rules. For example, different numbers of neurons could be generated in each subunit, as well as different types of neurons and different proportions of inhibitory and excitatory neurons and astrocytes. A fold with a particular function in the neocortex could then connect with a specific fold in the cerebellum, completing the interacting circuit. Nevertheless, redundancy and duplication of function have been built into the cerebellum that minimize the consequences of local damage in adults. We propose that developmental regulatory mechanisms are in place to buffer the developmental processes from small injuries that occur. A question for the future is whether stem or progenitor cells in the developing or adult cerebellum can replace damaged neurons long after they are born and the progenitors no longer normally generate the cell type.

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Early Purkinje Cell Development and the Origins of Cerebellar Patterning

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Abstract This chapter explores the mechanisms that regulate Purkinje cell neurogenesis, revealing the finely timed contribution of many regulatory genes in the control of PC progenitor specification, proliferation, subtype differentiation, migration, and survival from the cerebellar primordium to the end of prenatal embryogenesis, discussing some of the key molecules involved and the ways they combine to generate the complex adult cerebellar architecture.

Keywords Zebrin • Transverse zone • Stripe • Ventricular zone • Ebf2 • Reelin

PCs as Project Managers of Cerebellar Cytoarchitecture and Connectivity

The cerebellum contains a limited number of cellular phenotypes, arranged in a highly conserved circuitry and identified by their morphological features, their reciprocal relationships, and the expression of distinctive neurochemical markers. The mouse is the main model system in which cerebellar ontogenesis has been studied extensively. Although the mammalian cerebellum is superficially homogeneous, it actually consists of several hundred distinct compartments, which form a complex, reproducible array of transverse zones and parasagittal stripes. Cerebellar architecture is built around multiple Purkinje cell subtypes [1–6] – most notably zebrin II/

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aldolase C [7, 8] – which form the transverse zone-and-parasagittal stripe scaffold upon which the adult cerebellum is built. For example, zone-and-stripe boundaries restrict the terminal fields of many cerebellar afferent projections (reviewed in [9]), interneuron neurites [10] and somata (reviewed in [11]), and glial gene expression profiles (e.g., 5′-nucleotidase [12]).

In the mouse, the general timeline of events that leads to cerebellar maturation from its embryonic *anlage* has been fully clarified [13–18]. Here we discuss some of the major features of cerebellar development, focusing on the ontogenesis of Purkinje cells (PCs), the sole projection neurons of the cerebellar cortex.

PC development is only partially characterized, despite the remarkable progress made in recent years (reviewed in [19]). Achieving a better understanding of PC cell fate specification and ontogenesis in general is important for a number of reasons. First, PCs orchestrate the early stages of cerebellar development, namely those that precede the massive proliferation of granule cell precursors in the external granular layer. Only later in embryogenesis, and especially after birth, do granule cells take control of cerebellar histogenesis and foliation, as they outnumber all other cerebellar cell types by several orders of magnitude.

Secondly, PCs actually control granule cell clonal expansion by releasing the extracellular morphogen/mitogen sonic hedgehog [20–23], with the result that the overall PC number heavily influences the final dimensions and organization of the cerebellum – and ultimately its function. The corollary is that defective PC genesis or migration impairs granule cell clonal expansion, and cerebellar foliation/PC migration failures result in a lissiform adult cerebellar cortex: e.g., the naturally occurring mouse mutant *reeler* (Relnrl: reviewed in [24]).

Thirdly, PCs guide the wiring of the cerebellum. Most afferent fiber systems invade the cerebellum at around embryonic day 13/14 (E13/14) in the mouse [25, 26] and terminate with a spatial organization that parallels the pattern of PC stripes [27]. PCs instruct afferent fibers, including olivocerebellar axons, which eventually establish a one-to-one contact with their target, as well as mossy fibers, which connect transiently with PCs and use PC-produced guidance cues prior to retracting and shaping their definitive synapses on granule cell dendrites (reviewed in [16, 28]). PC subtype organization is thought to play a key role in instructing circuit wiring into topographic maps: zone-and-stripe boundaries typically restrict the terminal fields of both cerebellar mossy fiber and climbing fiber afferent projections (reviewed in [9]), and interneuron neurites ([10], reviewed in [11]) and spontaneous and engineered mouse mutants with disrupted PC stripes have complementary alterations in the spatial arrangement of afferent terminals [29–31].

Cerebellar Anlagen and Germinal Zones

The cerebellum arises from a specialized region at the midbrain/hindbrain boundary [32–34]. In the mouse, at E8.5, the antagonistic interaction that takes place between homeobox genes *Otx2* and *Gbx2* defines the isthmic organizer region [35, 36], which controls the development of cerebellar structures via the secreted

morphogens FGF8 and WNT1 [16, 37, 38]. At this stage, the cerebellar primordium consists of two distinct and symmetric bulges thought to grow and fuse on the midline, eventually giving rise to the vermis, flanked by the two hemispheres [15]. Importantly, however, homotopic and isochronic quail-chick grafting experiments have clearly shown that the caudal part of the early midbrain vesicle generates the rostral and medial part of the prospective cerebellum [32, 39–42]. Thus, the anterior part of the prospective cerebellar vermis, instead of resulting from fusion of lateral cerebellar plates (His, 1889), likely originates from the caudal and alar portion of the mesencephalic vesicle [39].

Once a low-resolution map has been drawn, cerebellar histogenesis begins, starting at E9. Around E9.5, two germinal neuroepithelia emerge in the cerebellar primordium, abutting the opening of the fourth ventricle: (1) the rhombic lip (RL), located at the outer aspect of the cerebellar plate, adjacent to the roof plate (RP, dorsal), and (2) the ventricular zone (VZ), lining the lumen of the fourth ventricle (ventral). These stem cell/progenitor compartments may be identified by the region-specific expression of two genes encoding basic helix-loop-helix transcription factors: pancreas transcription factor 1a (*Ptf1a*) in the VZ [43], and atonal homolog 1 (*Atoh1*) in the RL [44]. Cerebellar radial glial progenitors [45] expressing *Ptf1a* are fated to generate all GABAergic neurons of the cerebellum, including PCs and all inhibitory interneurons – cerebellar nuclear interneurons plus basket, stellate, Golgi, and Lugaro cells in the cerebellar cortex [43, 46, 47]. Homozygous mutations of *PTF1A* are associated with cerebellar agenesis in humans [48]. Conversely, all glutamatergic lineages – the large projection neurons of the cerebellar nuclei, unipolar brush cells, and granule cells – derive from *Atoh1*$^{+}$ progenitors [49–54]: their development is exhaustively reviewed elsewhere [19].

Important genetic networks involved in the maintenance of the stem cell/progenitor pool and in cell fate specification are active in the VZ and/or RL between E10 and E13. The stem cell marker SOX2 is expressed in both neurogenic territories (VZ and RL) and in the RP [55]. Its homolog SOX9 is largely co-expressed with SOX2 and may mediate termination of neurogenesis, thereby regulating a neurogenic-to-gliogenic fate switch in the mouse cerebellar primordium [55]. The target of Notch signaling, *Hes5*, is expressed in the VZ and RL, with a very sharp boundary and no expression in the RP. However, *Hes1* expression levels are low to absent in the VZ and RL but present in the RP [56, 57]. Notch1 in the cerebellar primordium interferes with BMP2/BMP4 signal transduction causing downregulation of the BMP target *Msx2*.

As shown by birthdating studies, cerebellar projection neurons, (PCs in the cerebellar cortex and glutamatergic neurons in the cerebellar nuclei), are born first, at the outset of cerebellar neurogenesis, while both inhibitory and excitatory interneurons are generated perinatally [15, 58, 59]. Dividing VZ precursors delaminate into the cerebellar presumptive white matter, while those of the RL migrate below the pial surface where they form the rhombic lip migratory stream, initially containing nucleofugal neuron progenitors and, later, the granule cell precursors of the external granular layer. Postnatal neurogenesis continues in both regions through the third postnatal week, giving rise to GABAergic and glutamatergic interneurons, respectively [15, 17].

Establishment of Neurogenic Microdomains for GABAergic Progenitors

A schematic representation of microdomains present in the cerebellar VZ is provided in Fig. 1. All cerebellar GABAergic neurons originate in the VZ from *Ptf1a*$^+$ [43] and *Ascl1*$^+$ [60] progenitors according to a two-step sequence [17, 19]. First, projection neurons (nucleo-olivary neurons and PCs) are generated from stem cells that give rise to fate-committed precursor populations. The nucleo-olivary neurons are generated between E10.5 and E12.5 in the mouse. Next, starting around E11 and through E13.5, mitotic PC progenitors exit the cell cycle and layer on top of the VZ

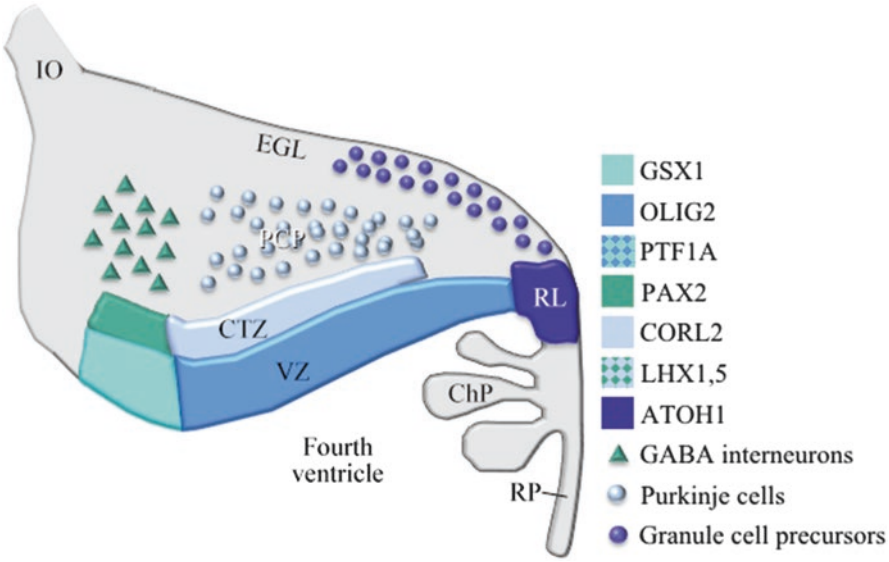

Fig. 1 A simplified representation of gene expression and cellular domains present in the E12.5 murine cerebellar primordium and giving rise to the mature cerebellar cortex. No reference is made here to cerebellar nuclei and their precurosrs. The drawing represents a sagittal section of the cerebellar anlage. The cerebellar primordium is bordered by the isthmic organizer (*IO*) rostrally and by the roof plate (*RP*) caudally. The choroid plexus (*ChP*), a roof plate derivative, is also shown. RP and ChP are non-neurogenic territories. The ventricular zone (*VZ*) is a mitotic cellular domain abutting the lumen of the fourth ventricle and giving rise to all GABAergic neurons of the cerebellar cortex. PTF1A is expressed by all GABAergic progenitors of the VZ, including PCs and interneurons. The PTF1A domain contains GSX1$^+$ cells (mitotic interneuron progenitors) and OLIG2$^+$ cells (mitotic PC progenitors). Both populations delaminate from the VZ (see text) giving rise to subventricular domains. The cortical transitory zone (CTZ) contains CORL2$^+$ potmitotic PC precursors that subsequently migrate into the Purkinje cell plate (PCP), underneath the external granular layer (EGL). GSX1$^+$ interneuron progenitors delaminate and give rise to PAX2$^+$ interneuron precursors fated to populate the prospective white matter (not shown) before homing into the cortex. Both PAX2$^+$ and CORL2$^+$ domains are also positive for LHX1 and LHX5. Finally, all glutamatergic neurons of the cerebellum originate from the rhombic lip (RL, positive for the proneural gene *Atoh1*). Among them, granule cell precursors migrate tangentially beneath the pia mater and populate the prospective EGL

to populate the nascent PC plate. The GABAergic interneurons are first born around E11 and sequentially generate all inhibitory local circuit neurons of the mature cerebellum.

The VZ is subdivided into mitotic progenitor domains abutting the ventricular lumen and corresponding postmitotic domains in the cerebellar primordium (an additional microdomain defines the rhombic lip) [61]. A microdomain positive for PTF1A contains two genetically defined progenitor cell types: OLIG2$^+$ PC progenitors occupy a more caudal position and undergo their terminal mitosis between E11 and E13; GSX1$^+$ progenitors are located more rostrally and medially. At E12.5, corresponding to the peak of PC neurogenesis, the c2 territory can be subdivided into a more caudal microdomain positive for CORL2, a selective marker of postmitotic PC precursors, and into a rostral/medial microdomain containing PAX2$^+$ interneuron precursors. PC precursors, after leaving the cell cycle, start migrating and populate different regions of the cerebellar cortex according to their birthdate [15, 52]. Instead, actively proliferating interneuron progenitors (IPs), positive for GSX1, begin to delaminate from the VZ giving rise to PAX2$^+$ interneuron progenitors and then migrate in successive waves to the nascent cerebellar nuclei or, with an inside-out progression, to the granular and molecular layers of the cerebellar cortex, where they acquire their definitive identities under the influence of instructive environmental cues ([62], reviewed in [63]).

The Regulation of PC Progenitor Specification and Commitment

At early stages (E11–12.5), a small number of GSX1$^+$ interneuron precursors are found in the most rostral region of the VZ, while the majority of PC progenitors occupy more caudal regions of the VZ. Ablation of *Olig2* has only a small effect [64] or no effect on PC number. However, a null mutation of both *Olig2* and *Olig1* produces a reduction of committed PC precursors [46]. As development proceeds, PC progenitors progressively become interneuron precursors, which spread from rostral (close to the isthmic organizer) to caudal, at the boundary between RL and RP. This temporal identity transition of cerebellar GABAergic neuron progenitors from PC progenitors to interneuron precursors is negatively regulated by OLIG2 and positively by GSX1 [46]. However, this view is challenged by the results of short- and long-term lineage tracing studies performed by other authors [64], suggesting that *Olig2*$^+$ progenitors may not contribute importantly to the interneuron precursor lineage. Further analyses will be required to resolve this discrepancy: one possible scenario is that *Gsx1*$^+$ progenitors affect the number of PC-committed *Olig2*$^+$ precursors (or the maintenance of the PC-committed stem cell pool) through a paracrine, non-cell-autonomous mechanism.

The VZ subregion containing PC progenitors is also characterized by the strong expression of E-cadherin (encoded by *Cdh1*) and of the cell surface marker NEPH3, which is a direct downstream target gene of PTF1a [65]. When OLIG2$^+$ PC progenitors

exit the cell cycle, they activate the expression of *Corl2* [66], which encodes a transcriptional repressor [67], and that of *Lhx1* and *Lhx5* [68], encoding LIM homeobox domain proteins. However, unlike CORL2, LHX1 and LHX5 label delaminating interneuron precursors as well as postmitotic PC precursors [46, 66]. Cells co-expressing LHX1/LHX5 [68] and CORL2 [66] are *bona fide* differentiating VZ-born precursors committed to a PC fate.

Other PTF1A targets are expressed in the VZ in addition to those described above [69]. The *Drosophila atonal* homologs *neurogenin 1* and *neurogenin 2* are proneural genes encoding basic helix-loop-helix transcription factors. *Neurog1*$^{+}$ progenitors give rise to inhibitory cortical interneurons and some PCs [70, 71], while *Neurog2* is expressed mainly in the PC- and presumptive nucleo-olivary neuron lineages. NEUROG2 controls progenitor cell cycle progression, promotes cell cycle exit and differentiation, and spurs the cell-autonomous phase of PC precursor dendritogenesis. Nullisomy for *Neurog2* causes a reduction in the overall PC number [72]. However, NEUROG1 and NEUROG2 are not required for the adoption of a PC fate (R. Hawkes, unpublished observation, and [72]). Interestingly, cell cycle analysis conducted by cumulative S-phase labeling on *Neurog2*^{CreERT2} knockin mice has revealed for the first time that at the peak of PC neurogenesis (E12.5), dividing VZ progenitors cycle in ∼14 h, and their basal-to-apical oscillating motion is compatible with interkinetic nuclear migration, similar to what has been shown in other territories of the neural tube, but never before in the cerebellar primordium [72].

Ebf2 and PC Subtype Specification

Thus far we have treated PC development as though all PCs are the same. This is far from the case – indeed in the adult mouse cerebellum, multiple PC subtypes have been identified (e.g., zebrin II/aldolase C [7]; PLCβ3/4 [73]; HSP25 [74]: reviewed in [6]). The embryological origins of PC heterogeneity and pattern formation are only slowly coming into focus. PC subtype phenotype is cerebellum intrinsic and independent of neural activity (e.g., [75]) or afferent innervation [76, 77]. Cerebellar compartmentation appears to start at ~E10 in the VZ of the fourth ventricle but likely not sooner (e.g., [78–81]). The first stage likely occurs when PCs undergo terminal mitosis between E10 and E13 [58] in the *Ptf1a*-expressing progenitor domain of the VZ [43, 69]. Birthdating studies have identified two distinct PC populations: an early-born cohort (E10–E11.5) fated to become zebrin II^{+} and a late-born cohort (E11.5–E13) fated to become zebrin II^{-} [82, 83]. However, individual PC stripes do not have a clonal origin [80]. There is also a direct correlation between PC birthdates and their adult stripe location, suggesting that both subtype specification and positional information (i.e., which zone or stripe the PC will occupy) may be acquired at this time (e.g., [82, 84–86]).

Several regulatory genes are implicated in PC progenitor development. Among them, *Early B-cell factor 2* (*Ebf2*) [87] belongs to a family of atypical basic helix-loop-helix transcription factors that do not possess a basic domain and instead

feature a unique DNA-binding domain. This family includes three transcriptional activators (EBF1–3) and one repressor (EBF4) (reviewed in [88, 89]). *Ebf2* is expressed in a subset of late-born PC progenitors fated to populate zebrin II$^-$ parasagittal stripes, and in *Ebf2* null mutants the cerebellum features a selective loss of zebrin II$^-$ PCs.

Upon cell cycle exit, late-born PC progenitors start expressing *Ebf2* and migrate toward the PC plate. Posterior-born PCs migrate tangentially at first and then follow radial glial fibers, projecting their axons ventrally into the prospective white matter [90]. Conversely, anteriorly born PCs migrate radially into the PC plate, also following radial glial fibers, to populate anterior regions of the cerebellar cortex. Migration of this latter population is reelin (RELN) dependent and selectively delayed in *Ebf2* null PCs, which accumulate before birth as an ectopic layer just above the VZ in the anterior third of the cerebellar anlage. A significant fraction of these PCs, many of which express neurogranin [91], dies by apoptosis [92, 93]. *Ebf2* is required to support survival of late-born PCs at birth and accomplishes this by transactivating the *insulin-like growth factor* (*Igf1*) gene. In postnatal *Ebf2* null cerebella, *Igf1* expression is downregulated, with a resulting impairment of IGF-1 signal transduction [93]. Finally, some of the *Ebf2* null PCs that survive lose their PC subtype specification features and trans-differentiate into zebrin II$^+$ PCs – the only genetic manipulation thus far shown to subvert PC subtype specification [92]. In fact, *Ebf2* acts to repress the zebrin II$^+$ phenotype in late-born PCs [85]. Further studies, employing conditional mutants, are required to determine at which stage of postmitotic PC precursor development EBF2 acts to specify PC subtype. The results of genetic fate mapping experiments (GGC et al.: unpublished data) suggest that *Ebf2* is expressed transiently in all PC progenitors, only to be restricted to late-born ones by the end of embryogenesis. The pathways that lead to further subtype specification (e.g., the HSP25$^{+/-}$ distinction within the zebrin II$^+$ family: [74]) have not yet been explored.

Embryonic PC Cluster Formation

Newborn PCs migrate dorsally into the cerebellar anlage where they aggregate by ~E17 into a reproducible array of clusters that already contains multiple distinct molecular PC phenotypes ([6], reviewed in [83], e.g., Fig. 2 [94–97]). These clusters are the targets by which cerebellar afferents and many interneurons become topographically ordered (reviewed in [5, 28]).

The mechanism that converts the PC plate into the elaborate array of embryonic PC clusters – >50 are recorded [97, 98] – is not well understood. As PCs migrate toward the cerebellar surface, the early-born (E10–E11.5: *Ebf2*$^-$; future zebrin II$^+$) PC lamina interdigitates with the more superficial late-born (E11.5–E13; *Ebf2*$^+$; future zebrin II$^-$) layer with the result that the stereotyped array of clusters emerges. Whether this migration is the mechanism that specifies cluster architecture or whether the clusters are already specified in the cerebellar plate (or are even preformed in the

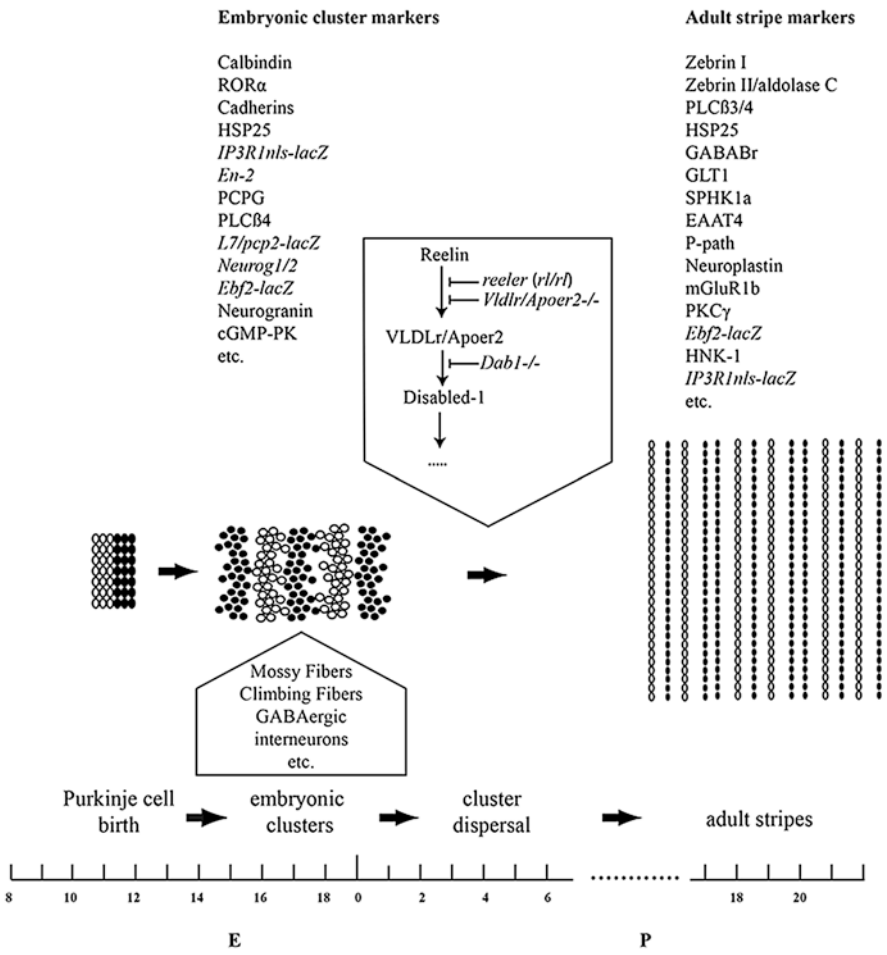

Fig. 2 From clusters to stripes. Embryonic clusters condense by migration from the cerebellar plate between E14 and E18 (mouse). At this stage numerous expression markers reveal that the PC population is already heterogeneous (exactly how many distinct phenotypes are present is not known, in part because of the paucity of double-labeling studies). The embryonic PC clusters also serve as a staging area to amass, organize, and restrict cerebellar afferents and interneurons. Starting perinatally signals via the RELN-Dab1 pathway trigger cluster dispersal into the adult cluster array by about P20. As for the embryonic clusters, the exact number of stripe phenotypes is not certain – at least ten may be identified based on expression data and mutant phenotypes. References to the lists of embryonic and adult cerebellar compartment markers may be found in [6]

VZ, i.e., some version of the protomap model proposed by Rakic for the neocortex: reviewed in [99]) is not known. The cellular processes that guide cluster formation are not understood, but grafts of dissociated PCs also organize into discrete, ectopic zebrin II^{+}/zebrin II^{-} aggregates [100], pointing to cell-cell adhesion molecules as possible organizers: cadherins (reviewed in [101]) and integrins (e.g., [102]) are

possible candidates. Also during this same period, the cerebellar anlage undergoes a 90° rotation, which converts the embryonic rostrocaudal axis into the mediolateral axis of the cerebellar primordium [81], so perhaps the adult stripe array ultimately derives from the anteroposterior patterning of dorsal rhombomere 1.

From Clusters to Zones and Stripes

Boundaries running from medial to lateral divide the cerebellar cortex into transverse zones. By combining different sources of evidence – molecular, genetic, and hodological – four highly conserved transverse boundaries, and hence five transverse zones, have been delineated in the adult mouse vermis (e.g., [103–105]): the anterior zone (AZ: ~ lobules I–V: reviewed in [104]), central zone anterior (CZa: ~lobule VI) and posterior (CZp), the posterior zone (PZ: ~lobules VIII to dorsal IX: reviewed in [105]), and the nodular zone (NZ). Each transverse zone is then further subdivided into a reproducible array of parasagittal stripes (e.g., revealed by using zebrin II/aldolase C [7, 8]: for zebrin II$^{+/-}$ stripes, these are labeled P^{+} and P^{-}, e.g., zebrin II [106], phospholipase (PL) Cß3/Cß4 [73]), the small heat shock protein HSP25 [74], or *L7/pcp2-lacZ* transgene expression (reviewed in [6, 103]).

PC stripes are discontinuous across transverse boundaries so it seems plausible that the zones precede stripes in development, but whether transverse zones form prior to the PC clusters or at the same time is speculative. Transverse boundaries are certainly present in the embryonic cerebellum. The AZ/CZa boundary between lobules V and VI can be identified both in neonates and adults by the expression domains of numerous molecules (e.g., calbindin [103], reviewed in [107]) and is a developmental phenotype restriction boundary for several cerebellar mutations. (In some cases, the mutant phenotype is associated with defects in the AZ (e.g., [108]), lurcher Grid2Lc-J: [109], and *cerebellar-deficient folia* (Ctnna2cdf: [110]); in others – for example, the BETA2/*NeuroD1* null [111] – it is the posterior cerebellar zones that are the most affected.) Finally, a granular layer lineage restriction boundary also lies in the anterior face of lobule VI, indicating that granule cells either side of the boundary derive from different lineages [112]. The CZa/CZp boundary [113] is a perinatal restriction boundary for FoxP2 [107], *Gli* [95], and HNK-1 expression [114]. The CZp/PZ boundary that separates lobule VII from lobule VIII is revealed in the perinatal cerebellum by FoxP2 [98, 107], PLCβ4 [115] and HSP25 [116] expression which is associated with a phenotypic abnormality in the *lurcher* (*Grid*^{Lc}) mouse [109]. Finally, the most caudal transverse boundary in the adult mouse (PZ/NZ) lies near the base of the posterolateral fissure between lobules IX and X. A transverse boundary has also been located in the same area during development as a restriction boundary for the expression of En2 [95] and FoxP2 [96]. A granular layer transverse boundary in embryonic stem cell chimeras is also located at around the PZ/NZ boundary [112].

Starting at around E18, the embryonic clusters transform into adult stripes triggered by RELN signaling (reviewed in [117, 118]). Because PC dispersal and the

associated development of cerebellar foliation occur almost entirely along the rostrocaudal axis, each cluster becomes stretched out into a long, narrow stripe.

RELN is secreted by both the external granular layer and glutamatergic projection neurons of the cerebellar nuclei [117] and binds to two PC receptors – the apolipoprotein E receptor 2 (Apoer2) and the very low-density lipoprotein receptor (VLDLR: [119, 120]). Both receptors are required for normal stripe formation, and if RELN is absent (e.g., the *reeler* mouse (*Reln*^{rl})), PC cluster dispersal is blocked, and the adult mouse retains the embryonic cluster morphology and is ataxic (reviewed in [24]). RELN binding induces Apoer2/Vldlr receptor clustering [121], which triggers a protein kinase cascade and tyrosine phosphorylation of the docking protein Disabled1 (DAB: [122–126]) by Fyn and Src [127, 128], leading eventually to a drop in mutual PC-PC adhesion, possibly via integrins. In parallel, DAB1 phosphorylation also activates Rac and Rho GTPases, which control actin filament assembly [129]. Together cytoskeletal and cell adhesion changes are thought to permit the embryonic PC clusters to disperse into stripes. That being said, it is not clear whether cluster dispersal requires the active migration of PCs or is the passive consequence of lobule formation.

However, the RELN pathway is not that straightforward. First, while expression mapping suggests that all PCs express both Apoer2 and Vldlr RELN receptors, mutations in individual receptors (*Apoer2*$^{-/-}$ and *Vldlr*$^{-/-}$ nulls; *Apoer2*$^{+/-}$:Vldlr$^{+/-}$ double heterozygotes) result in specific partial *reeler* phenotypes with some clusters dispersing normally, while others remain ectopic ([130]; divergent roles are also seen in the developing cerebral cortex [131]). Similar behavior is seen in several naturally occurring mutants. For example, *meander tail* (mea2J [132]), *rostral cerebellar malformation* (Unc5crcm [108]), 1998), and *cerebellar-deficient folia* (Ctnna2cdf [110]) all display selective PC ectopias that are restricted to the zebrin II^{-} phenotype (and because zebrin II^{-} PCs are preferentially located in the AZ, it is the anterior vermis that is most severely affected). In a more complex model – the *weaver* (*Kcnj6*^{wv}) mouse – PC cluster dispersal failure is restricted to zebrin II^{+}/HSP25^{+} stripes in the CZa/CZp [133]. The GIRK2 protein mutated in *weaver* [134] is expressed by all PCs so the molecular basis of the selective PC ectopias is unknown.

The relationship between the embryonic cluster topography and the zone and stripe pattern of the adult is not fully mapped. Because a few markers are expressed consistently in both clusters and stripes (FOXP2: [98], several are, for example, PLCß4 [115], an IP3R1 promoter-nls-lacZ transgene [135]), but others are only expressed in stripes at one stage or show very different expression patterns perinatally versus the adult, e.g., HSP25 (e.g., HSP25 [116], lysosomal acid phosphatase 2 [136]), there is limited evidence of the continuity of the cerebellar topographic map from perinate to adult. In theory, three relationships might occur: one embryonic cluster might form a single adult stripe; one cluster might split to yield more than one stripe; or several clusters might combine into a single stripe (Fig. 3). In fact, all three possibilities have been described. In several cases, the one cluster = one stripe model seems very likely (e.g., [83, 98, 135]). However, other examples are more complex. For example, the so-called P1^{-} stripe in the AZ vermis clearly derives from three distinct embryonic clusters, which abut (as revealed by using

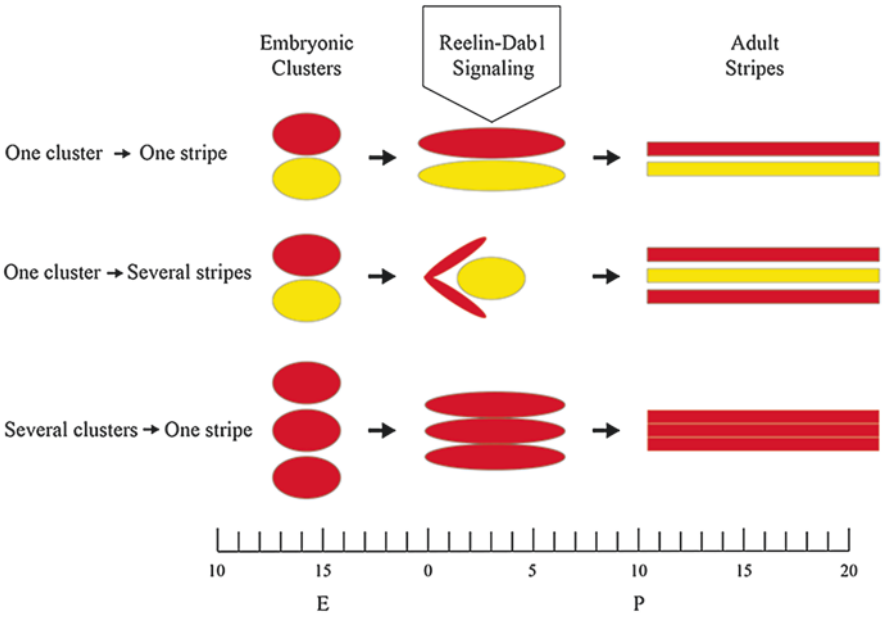

Fig. 3 Three models for the transformation of embryonic PC clusters into adult stripes: one embryonic cluster forms one adult stripe; one cluster splits to yield several stripes; or several clusters combine into a single stripe. All three models are found

PLCß4 expression [115]). An alternative – and perhaps better – description is that the apparently homogeneous P1$^-$ stripe in the adult (all zebrin II$^-$/PLCß4$^+$) actually comprises three distinct sub-stripes. The triplet structure is also seen in the afferent mossy fiber projections (where cuneocerebellar and spinocerebellar pathways terminate in different sub-stripes: [137, 138]) and in the expression of an *L7/pcp2-lacZ* transgene [103]. A similar covert heterogeneity is seen in ostensibly homogeneous zebrin II$^+$ stripes when co-labeled for HSP25 [74]. Last, single clusters may give rise to multiple stripes. For example, inducible fate mapping with a *Pcp2-CreER-IRES-hAP* transgene showed three cluster pairs contribute to nine adult stripes [28].

Finally, a striking feature of adult cerebellar topography is the high reproducibility between individuals and the concomitant low error rate (e.g., zebrin II$^+$ PCs are very rarely seen in zebrin II$^-$ stripes). If stripes derive from clusters, and stripes have no errors, then either clusters have no errors (and migration from the VZ to the clusters is perfect) or errors that occur during cluster formation and dispersal are selectively eliminated. In this context it is interesting that many PCs – perhaps as many as a third – undergo cell death by apoptosis during the perinatal period [139]. This suggests the hypothesis that perinatal apoptosis eliminates those PCs that wind up in the wrong embryonic cluster. PC ectopia is not lethal per se: for example, PCs located ectopically may survive indefinitely. Rather, the hypothesis evokes a community effect, such that being in the wrong cluster leads to apoptosis. In support of the idea that apoptosis

refines topography, studies of naturally occurring cell death in the cerebellum identified hot spots of PC apoptosis that correlate with stripe boundaries in the adult [140]. However, preliminary experiments do not support the hypothesis. Deleting the Bcl-2-/BH3-associated apoptotic protein BAX inhibits perinatal PC death (BAX is expressed in PCs perinatally [141] and *Bax−/−* mice have a 30% excess of PCs over controls (e.g., [142]). Nevertheless, the frequency of targeting errors was unaffected (RH and Y. Wang: unpublished data). Therefore, the remarkable reproducibility of the cerebellar map does not seem to result from perinatal error correction.

Conclusions

Early stages of PC development affect both susceptibility and outcome of several motor and cognitive disorders. Cerebellar development is protracted (from E7 to P30) and complex (at least two germinal zones, multiple migration pathways, etc.) so it is unsurprising that it represents a large target for developmental disruption. Spinocerebellar ataxia type 1 provides an example of this: transgenic mice in which expression of the expanded *ATXN1* transgene is delayed until after the cerebellum has matured display a reduced disease phenotype, suggesting that mutant *ATXN1* interacts with a pathway involved in PC development, likely by affecting *RORa* expression. Thus, compromising PC development appears to contribute to the severity of neurodegeneration [143]. Equally striking, recent evidence has linked PC development to the pathogenesis of autistic spectrum disorders (reviewed in [144]). In particular, selective deletion of the *Tsc1* gene in the PC lineage from conditional knockout mice has been found to cause a decrease in PC number, increased spine density, and autistic-like alterations of social behavior [145]. One of many insults thought to trigger autism is maternal fever [146]. Possibly related to the putative role of the cerebellum in autism, we recently found that immune activation and fever in pregnant mice between E13 and E15 resulted in adult progeny with significantly wider zebrin II$^{+/-}$ stripes, greater numbers of PCs, poorer motor performance, and impaired social interactions in adolescence [147].

Finally, what are the prospects that early intervention might afford therapeutic advantages? While fast progress has been made in recent years, plenty remains to be learned in regard to the signals that instruct VZ progenitors to adopt PC versus GABAergic interneuron fate. To our knowledge, protocols aimed at producing PCs from ES/iPS cells in vitro are based on selection of early PC progenitors that express lineage-specific surface markers [148]. The identification of additional factors cooperating with PTF1a and OLIG2 in specifying the earliest PC progenitors should improve the efficiency of those protocols and make it possible to generate autologous PCs from iPS cells or via direct reprogramming. These short-range projection neurons produced in vitro may eventually constitute a source of cell replacement in patients affected by certain types of degenerative ataxias.

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Cerebellar Developmental Disorders and Cerebellar Nuclei

Hong-Ting Prekop, Alessio Delogu, and Richard J.T. Wingate

Abstract While significant progress has been made in the last 10 years in understanding the development of cerebellar nuclei, they remain a relatively less well-studied cell group in the brain. In this chapter, we review the anatomical organisation of the cerebellar nuclei and their connections to highlight outstanding developmental questions. We then describe recent progress in dissecting the lineages of cerebellar neurons that may point to new understanding of their involvement in congenital clinical disorders.

Keywords Dentate nucleus • Interposed nucleus • Fastigial nucleus • Inferior olive • Purkinje cell • Rhombic lip • Ventricular zone • Ptf1a • Atoh1 • Pax2 • Nuclear transitory zone

What Are Cerebellar Nuclei?

The cerebellar nuclei (CN) are the final output units for cerebellar processing. For the most part, the CN output is a high-frequency tonic excitation, which is directed towards the midbrain and thalamus. However, a distinct, long-range inhibitory axon tract allows the CN to influence the activity of the inferior olive (IO), which in turn drives Purkinje cell (PC) activity via climbing fibres. CN output is modulated by the patterned firing of inhibitory PCs. They thus form the final common pathway for the integrated activity of a series of nested re-entrant loops via the inferior olive but also via the thalamus, cortex and pons (Fig. 1).

Despite the central position of CN within these major long-range networks, relatively little is known about their component cell types, the synaptic arrangement of their component interneurons or their processing role. Their development has only

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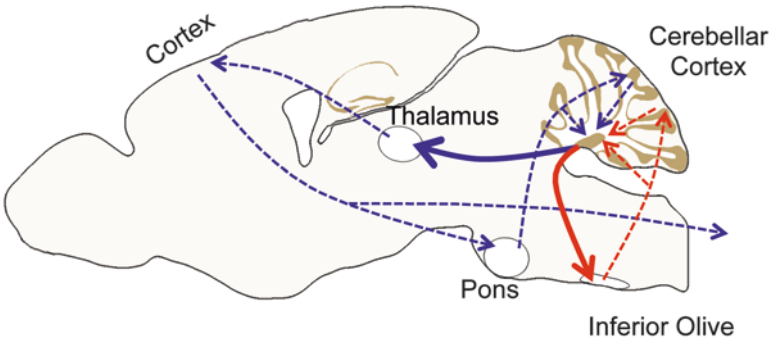

Fig. 1 The cerebellar nuclei are central to cerebellar circuitry. They lie at the centre of two cerebellar loops: the cerebello-thalamo-cerebro-cortical circuit (*blue*) which link the cerebellum back to the cerebral cortex and the olivo-cortico-nucleo-olivary loop (*red*)

recently been described, and, even then, the picture is partial. Major questions remain as to how nuclei achieve their spatial arrangement, integrate cell types of different origins and make connections. For a population of such significance for a wide variety of brain functions, this is a major omission. Similarly, while some nuclear disorders in humans have been described, the lack of anatomical and molecular description has hampered a systematic analysis of clinical disorders.

Cellular Anatomy and Diversity

The earliest descriptions of CN neurons distinguished cells with long axons from those with short axons [1] and identified large and small soma size [2]. The most detailed morphological studies of the rat and primate dentate (lateral) cerebellar nucleus were carried out by Victoria Chan-Palay in the 1970s. Using Golgi, Nissl and Weigert preparations combined with electron microscopy, she mapped out the complex, non-uniform cellular organisation of the nucleus [3–5] and demonstrated the presence of two types of projection neurons with at least three different types of cells with short axons and small soma. These latter neurons were designated as local interneurons on the basis of dendrite and axon morphology and could be distinguished by their multipolarity or bipolarity and fusiform soma.

Immunohistological and molecular techniques have subsequently shown large projection neurons to be glutamatergic (projecting to the red nucleus, thalamus or brainstem), while projection neurons with very small soma that project to the inferior olive are GABAergic [6–8] (Fig. 2). In addition to these latter nucleo-olivary inhibitory projections, glycinergic neurons can project to both the brainstem [9] or to the granule cell layer of the cerebellar cortex [3, 10–12]. Unlike the other CN cell types, these latter nucleo-cortical neurons are not spontaneously active but instead are mostly silent. They most likely target Golgi interneurons, which express glycine receptors, unlike most cells of the granule cell layer [13].

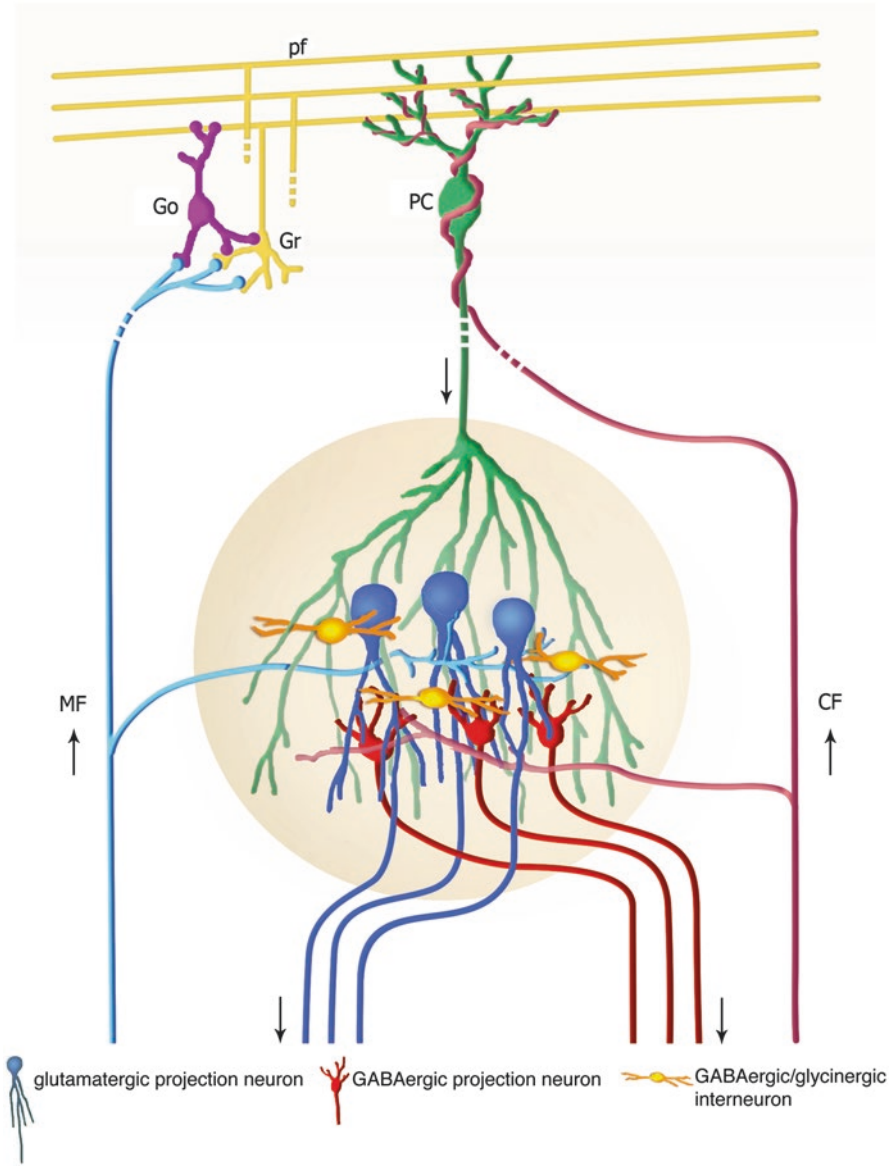

Fig. 2 The cellular composition of the cerebellar nuclei. Nuclei receive inputs from the Purkinje cells in the cerebellar cortex (*green*), as well as collaterals from the mossy fibres (*light blue*) and climbing fibres (*pink*) as they travel to the cortex. Within the nuclei, there are two types of projection neuron: large glutamatergic cells (*blue*), which are efferent cells in the cerebello-thalamo-cerebro-cortical circuits, and the nucleo-olivary neurons (*red*), which project to the inferior olive, forming the olivo-cerebellar loop. Interneurons (*orange*) participate in as yet uncharacterised local circuits

Other larger glycinergic projection neurons are found in the medial nuclei [14] and project ipsilaterally to the vestibular nuclei, the ventral brainstem and the ipsilateral ventromedial medullary reticular formation. These are hence the ipsilaterally projecting counterparts to the large glutamatergic neurons of the same region, which project contralaterally to the same regions. This has raised suggestions that posture and balance rely on a system of cross-midline control, similar system to that of the vestibular control of horizontal eye movements [15].

Relatively little is known about the local interneurons. Chan-Palay [4] noted small GABAergic neurons with fusiform or multipolar somas, limited dendritic trees and short axons, but it is possible that some of the cells observed could be the small nucleo-olivary neurons. A population of glycinergic neurons with small somata have also been found in the interposed and lateral nuclei. Because glycinergic terminals are found mainly on adjacent, presumptive glutamatergic projection neurons, it has been suggested that these are interneurons [14, 15], which colocalise with GABA [16]. GABAergic terminals that did not derive from PCs are also indicative of GABAergic interneurons or possibly local collaterals from the nucleo-olivary neurons. Though it is not possible to differentiate nucleo-olivary neurons from other GABAergic cell types in the CN based on size, there are some electrophysiological differences that aid identification [9].

Despite the fact that cells differ along both rostral-caudal and lateral-medial axes in terms of prevalence and dendritic/axonal trees, models of cerebellar function assume a homogeneous spread of each CN cell type, paralleling the long-assumed homogenous and stereotyped circuitry of the cerebellar cortex, which itself is undergoing re-examination [17]. For example, there is a higher density of nucleo-olivary neurons in the ventral lateral and interposed CN [18]. Accordingly, the PC axon terminals spread in a different manner in these parts when compared to more dorsal and medial regions of the CN [19]. On the whole, the diversity, connectivity and processing function of local interneurons have remained elusive and thus disregarded in circuitry models.

The origins of CN, how their distribution is specified and how local circuits are set up and refined are all important questions that remain to be addressed. PCs can inhibit GABAergic CN neurons, so disinhibiting glutamatergic projection neurons through local networks.

Outputs of the Cerebellar Nuclei

The CN translate cerebellar output to the cerebral cortex via the thalamus, brainstem and spinal cord through two main long-range projection systems: glutamatergic projection neurons send signals to the red nucleus, thalamus, or brainstem, while the GABAergic nucleo-olivary neurons connect the cerebellum to the inferior olive [7]. Meanwhile, other forms of efferent connections have also been found linking the CN to the vestibular nuclei and the cerebellar cortex [10, 15].

Glutamatergic projection neurons form a vital link in the assorted cerebello-thalamo-cerebro-cortical circuits which link the cerebellum back to different parts of the cerebral cortex [20]. The nucleo-olivary neuronal projections are thought to form the olivo-cortico-nucleo-olivary (OCNO) loop, a closed feedback loop between the inferior olive, cerebellar cortex and CN, made up at a fine scale of individual closed loops, or cerebellar modules, of local connections via the CN [21]. While this closed loop model is challenged by the existence of bilaterally extending nucleo-olivary neurons [22, 23], it remains a compelling architecture to describe the functional properties of the cerebellar circuit.

The origins of the diversity and the mechanisms underlying the targeting of their axons are largely unexplored. Each of these characteristics is core to an understanding of how the cerebellum influences other parts of the brain.

Inputs to Cerebellar Nuclei

The inputs to the CN comprise a complex matrix that modulates cerebellar output by influencing the spontaneous baseline firing rate of CN neurons [24, 25]. The most significant of these inputs are PCs from cortical layers directly above the corresponding part of the CN: the medial receiving input from the vermis, interposed from paravermis and the lateral receiving the bulk of its input the hemispheric PCs [26]. Sugihara et al. mapped PC projections to the various CN and found correspondence between aldolase C expression in subsets of PCs and the terminations in specific subdivisions of CN, demonstrating some conservation of topographic organisation [27].

While both PCs and CN neurons are spontaneously active [28, 29], evidence of synaptic plasticity at the CN neurons shows that the CN are involved in modulating cerebellar cortical output and not merely relaying information from the PC population [30–32]. When PC and CN neurons are monitored simultaneously, they do not give the expected reciprocal firing rates that would result from PC inhibition [33–36]. Instead CN neurons are extremely sensitive to the synchronous activity of PC inputs [37] suggesting that the development of a mapping of PC populations into the CN is a critical factor in cerebellum function.

In addition to afferents from the PCs, the CN also receive collaterals from mossy fibres (MFs) and climbing fibres (CFs). These send signals directly to the CN, bypassing cerebellar cortical processing [26]. In the overlying cerebellar cortex, MFs and CFs are topographically mapped onto GCs and PCs, and their collateral projections to CN follow approximately the same topography. MFs from the pontine nuclei, nucleus reticularis tegmenti pontis and lateral reticular nucleus send their cortical terminations such that they divide the cerebellar cortex into zones to process information from particular parts of the body or sensory modes [23, 38, 39]. In contrast, the MF collaterals to the CN are bilateral and show a looser zonal organisation [26, 40]. Likewise, anterograde tracing from the inferior olive has revealed a strict topographic alignment of CFs to the zebrin II-positive PC parasagittal zones in the contralateral cerebellar cortex [19]. The collaterals of these same CFs target the contralateral CN and terminate in specific areas of the CN [27, 41, 42].

Relatively little is known of how inputs to the CN are organised at a cellular level and the intrinsic networks that are built up by interneurons and local collaterals. A natural entry point to these questions is trying to understand the degree of convergence of a relatively orderly PC layer on to the three-dimensional assembly of CN neurons. In terms of numbers, there are around 20 PC to every CN neuron [43, 44] with inputs targeting both glutamatergic [45, 46] and GABAergic projection neurons [8]. However, since the PC axonal target field is wide and conical [47], it is estimated that each PC can encompass tens of CN neurons complicating a simple explanation of convergence. Similarly the proximity of axon terminations to the soma of CN neurons is likely to be of considerable significance in determining synaptic strength [14]. Chan-Palay noted that around 14% of larger neurons in the lateral CN were not innervated directly on their somata by PCs, setting apart a subset of projections neurons [48], which may comprise the glycinergic, nucleo-cortical neurons [11].

How the PC axon numbers are developmentally matched to CN targets and the mechanisms that regulate mapping are unknown. Similarly, how the topography of collateral projections from different afferent populations is coordinated within the nucleus is an important question that remains to be addressed. For example, it has been suggested that collaterals of inputs to the cerebellar cortex form a template for topographic refinement of outputs of Purkinje cells to the CN.

Development of Cerebellar Nuclei

The origins of the cerebellum, which sits at the boundary of the midbrain and hindbrain, were an intensely investigated problem at the end of the last century. The advent of molecular techniques revised the concept that the cerebellum received contributions from both the midbrain and hindbrain and identified the cerebellar anlage within the dorsal part of rhombomere (r)1 of the hindbrain [49–51]. Within the anlage, two distinct progenitor zones, which are defined by the mutually exclusive expression of basic helix-loop-helix (bHLH) transcription factors Ptf1a and Atoh1, produce all the cell types of the cerebellum [52]. Ptf1a is expressed in the dorsal ventricular zone of r1 and characterises progenitors of GABAergic cells [53]. The boundary between the ventricular zone and the dorsal roof plate is known as the rhombic lip [54] and expresses Atoh1 [55]. This highly proliferative zone of Atoh1 induction gives rise to glutamatergic cerebellar neurons [56, 57].

Birthdating has shown that some neurons within the CN are among the first-born cell types of the cerebellum [58]. Experiments using either BrdU or a replication-defective adenovirus [59] have shown that PCs are born around the same time as the CN. The time window for the production of glutamatergic and the GABAergic projection neurons in mice lies between E10.75 and E12.5 [60] and appears to be regulated by a common temporal signal [61]. However, the allocation of GABAergic versus glutamatergic fate is strictly a property of progenitor position within either a Ptf1a- or Atoh1-positive pool [53, 56, 57, 61, 62].

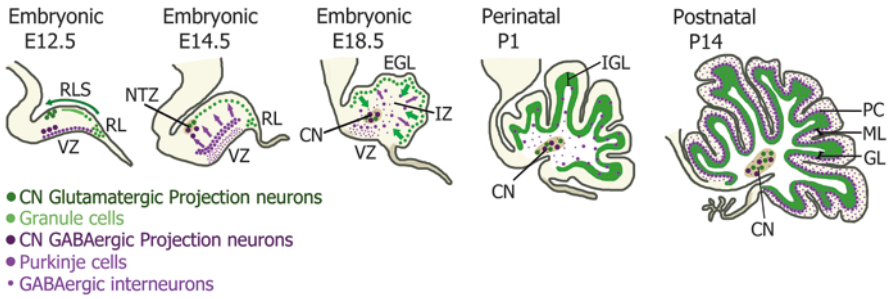

Fig. 3 The developmental timeline of the cerebellum, depicted in sagittal view. GABAergic neurons are derived from the ventricular zone (*VZ*), while glutamatergic neurons arise at the rhombic lip (*RL*). The cerebellar nucleus projection neurons are the first born from both progenitor zones, preceding first Purkinje cells (*VZ*-derived) and then granule cells (*RL*-derived). Cerebellar nucleus interneurons are believed to be born alongside other cerebellar cortical interneurons, which are generated from E13 from the *VZ* and later a stem cell population within the future white matter

Origin of Glutamatergic Neurons

One key motif of CN development is the assembly of neurons within an embryonic nuclear transitory zone (NTZ), which appears as almost a "staging post" in the formation of distinct CN (Fig. 3). The derivation of glutamatergic CN neurons initially appeared to be via a radial migration from the ventricular zone [63]. A detailed analysis of postmitotic precursors of CN neurons identified the expression of the transcription factors Lhx2/Lhx9, Meis 1, Meis 2 and Irx3, as well as genes that are not frequently used as markers in development: Gja9, Mbd2, Htr3a and Girk4 [64]. Subsequent analysis showed that Meis 2 co-expresses with Lhx2/Lhx9 in glutamatergic projection neurons of the lateral CN derived from the rhombic lip [57], while Irx3 may instead represent a separate population of neurons, likely the GABAergic nucleo-olivary neurons [65].

Glutamatergic projection neurons represent the first cohort in a sequence of neurogenesis from the rhombic lip that ends with the generation of granule cells [49, 56, 57]. A separate domain of Atoh1 expression at the midbrain-hindbrain boundary gives rise to earlier-born extracerebellar neurons [66]. At the rhombic lip, lateral and then medial CN are produced in discrete temporal waves [67, 68]. CN neurons actively migrate from the rhombic lip in a subpial layer guided by diffusible netrin and slit proteins [69, 70] and sequentially express Pax6, Tbr2, Tbr1 and Lmx1a [65, 71]. As the postmitotic neurons enter the NTZ, Tbr1 and Tbr2 are upregulated and Pax6 is downregulated [71]. In the absence of Pax6, rhombic lip-derived CN neurons are absent from the cerebellum [65]. The differential retention of transcription factors defines different CN populations in mouse. Tbr1 expression is retained until E14.5 for lateral and interposed CN and into adulthood for the medial CN. In contrast, the lateral and interposed CN projection neurons express Brn2 at early postnatal stages.

Origin of GABAergic Projection Neurons

The developmental origins of the GABAergic nucleo-olivary neurons are enigmatic. It is assumed that they are born from the ventricular zone like the other GABAergic cell types of the cerebellum, although direct evidence for this is lacking. Like the glutamatergic populations of the CN, GABAergic neurons are likely to arise as part of a discrete temporal window of cell production. It is thought that the GABAergic projection nucleo-olivary neurons are first in a ventricular zone temporal lineage (Kim et al. 2011) that subsequently gives rise to PCs (e10.5–e12.5 in mouse) followed by other GABAergic interneurons [72]. In contrast to these later-born cell types, both PCs and GABAergic CN neurons express Neurog2 [73]. Postmitotic cells expressing Neurog1 appear to be candidate CN nucleo-olivary projection neurons [74]. Irx3 immunopositive cells are evident in the VZ from E10.25 to E12.5, the NTZ at E13.5 and by E15.5 the cells have migrated into an intermediate zone outside the NTZ [64, 65]. Irx3 expression persists in the sey/sey ("small eye" pax6 null) cerebellum confirming that the specification of GABAergic and glutamatergic neurons is independent of each other.

Other GABAergic Neurons

VZ progenitors require the expression of Ptf1a for GABAergic specification [53, 62]. Within the Ptf1a ventricular zone, combinatorial gene expression demarcates discrete germ zones that are thought to give rise to the different types of interneurons [64, 72, 74–79]. Thus, for example, Neurog1 and Neurog2 expression defines subsets of the Ptf1a+ VZ population.

However, this topographic explanation of diversity is complicated by evidence that proliferation continues within a single population of Pax2+ precursors from the VZ [80] that persists in the prospective white matter well into postnatal development in mouse. Heterotopic and heterochronic grafting experiments have found that Pax2 progenitors generate all the remaining inhibitory interneurons [80, 81], including Neurog1 (Ngn1)-positive interneurons of the CN, which are born at E17.5 in mouse [82]. Mutation of PC progenitor transcription factors Olig2 and Gsx1 disrupts the production of Pax2 lineages suggesting that the latter is derived from the former in development [83]. The origin and development of the various types of glycinergic neurons in the CN have yet to be characterised.

Nucleogenesis and Cell Migration

The different developmental origins of different types of CN neurons require that cells recognise each other and assemble nuclei distant to their origins. How nucleogenesis – the migration, organisation and synaptogenesis of CN neurons – is organised is

unknown. Clearly, either intrinsic programming or cues in the surrounding environment or a combination of both will be key factors in this developmental process.

For rhombic lip derivatives, unipolar neuroblasts move within a subpial stream towards the NTZ guided by both diffusible netrin and slit [69, 70] (NTZ); however the cues that determine the position of the NTZ itself are unclear. One possible determinant is the underlying axon scaffold of the fasciculus uncinatus, to which first-born CN cells then contribute [67, 69]. Changing the fate of CN neuroblasts blurs the boundaries between distinct populations in the NTZ but does not compress or expand the map of presumptive CN. Thus when either Lhx9 (lateral CN in mouse) is overexpressed in chick [67] or Tbr1 knocked down in mouse [71], CN neuron number remains similar but boundaries are less discrete. From the NTZ, cells are then incorporated into the white matter through what might constitute an active radial migration or a passive translocation as a consequence of the overall pattern of cerebellar morphogenesis [60, 63].

Evidence in favour of radial migration being a component of nucleogenesis comes from the analysis of the reeler mouse. Pax6/reelin-positive neuroblasts migrate from the rhombic lip, and at least some go on to become Tbr2-positive CN neurons. The reeler mouse has disrupted CN architecture; however, the initial tangential migration of rhombic lip derivatives to the NTZ is normal [71].

Evolution and the Diversification of Cerebellar Nuclei

While some aspects of the cerebellar circuit are among the most evolutionarily conserved across vertebrates, cerebellar nuclei are relatively variable in composition [84]. There is some debate over whether an organism is considered to have cerebelloid structures if they lack CN, since it is these cells that form the dominant output [85]. For example, teleost fish have no white matter or CN. Instead, their PCs project to eurydendroid cells, which then project to other parts of the brain. However, eurydendroid cells also receive inputs from granule cells via parallel fibres and are found within the granule cell layer and so are not homologous to CN projection neurons in terms of inputs [86, 87].

The replacement of CN by eurydendroid cells appears to be a ray-finned fish adaptation as there is evidence for a single cerebellar nucleus in the shark [88]. CN are absent in lampreys, where the cerebellum is reduced or absent. Across fish species the medial and dorsal octavolateral nuclei receive inputs from lateral line systems and are involved in spatial calculations that are analogous to those carried out in the cerebellum. It seems conceivable, though yet to be proved, that these may be considered as ontological homologues of CN [89].

Like sharks, amphibians have a single CN; however the number and diversity of CN increases in amniotes. There are two CN in birds [90] and three sets of CN in rodents: the medial, interpositus and lateral [91, 92]. In cats, rabbits and primates, there are four major CN: the medial, or fastigial, nucleus; the anterior and posterior interposed and the lateral, or dentate, nucleus. Each of these nuclei can be functionally

further subdivided such that complexity of CN organisation is a marked feature of mammalian brains [14]. This systematic variation in organisation suggests that comparative studies may offer an important insight into the significant genetic factors in the development of CN diversity.

Cerebellar Nuclei and Disease

The relatively recent discoveries of the developmental lineages of CN neurons highlight previously unexplored relationships in cerebellar disorders and disease. Glutamatergic projection neurons are formed from Atoh1 progenitors that not only generate granule cells but also neurons in the pons, vestibular and auditory systems of the hindbrain [57, 93]. GABAergic neurons share a progenitor transcriptional profile with auditory nuclei and, perhaps most prominently, the inferior olive [53].

This is particularly significant in that developmental disorders where cerebellar nucleus exclusively malformed have not been reported. Congenital dysplasia of the dentate and olivary nuclei (DOD), though rarely recorded [94], can sometimes be detected as a minor pathology of more extensive developmental defects (Table 1). Though pathogenesis may differ across different forms of DOD, it is interesting to note that many of the below conditions have pathologies of the inferior olive too. While the correlation in pathologies could be linked by lineage, the possibility of retrograde degeneration of the cerebellar nucleus as a result of inferior olive dysplasia cannot be discounted. Similarly, the possibility that the modularity of the cerebellar-inferior olive closed loop extends to a single cell level [95] means that heavily interconnected microzones might suffer a conductive degeneration when any element of the system is disrupted.

While DOD might represent a failure of Ptf1a lineage development, pontocerebellar dysplasia might conversely reflect a dysgenesis of Atoh1 lineage neurons, affecting both precerebellar and granule cell populations in addition to portions of the dentate CN. In both cases, the spectrum of associated phenotypes raises the possibility of a developmental origin within the specification or maturation of specific populations of derivatives.

Future Perspectives on Cerebellar Nucleus Development

In recent years, significant progress has been made with regard to understanding the development of the glutamatergic CN neurons, while physiologically, models of cerebellar function increasingly recognise how plasticity and modulation within the CN by mossy fibre and climbing fibre collaterals place these cells at the heart of cerebellar networks [43, 115]. However, less is known of other, equally significant, CN neuronal types and key questions about their specification and lineage remain

Table 1 Cerebellar disorders exhibiting nuclear pathology

Disorder	Aetiology	Pathology	Clinical features	Reference
Zellweger (cerebro-hepato-renal) syndrome	Autosomal recessive disease caused by mutations in PEX genes. Migration failure from 14 weeks of gestation in humans	Dysplasia of the dentate and olivary nuclei (DOD), as well as cerebellar hypoplasia and migrational defects of PCs	Developmental delay, seizures and EEG abnormalities, as well as generalised hypotonia, renal cysts and joint calcifications	[96–98]
Dentato-olivary dysplasia with intractable seizures in infancy	Unknown, though suggested to be autosomal recessively inherited	DOD – The dentate nuclei are seen as a solid ovoid or tear-shaped structure rather than the characteristic thin, convoluted band	Hypotonia with frequent seizures from birth and gross developmental delays. Survival is no longer than 3 years	[99, 100]
Joubert syndrome	Autosomal recessive disease approximately 50% of cases are genetically linked to mutations in genes that encode parts of the primary cilia. These may be important in progenitor cells for sensing morphogens like Wnt and Shh during development	Fragmentation of the dentate CN as one of many hindbrain symptoms, along with hypoplasia of vermis (molar tooth sign), dysplasia of the inferior olive and non-decussation of the SCP	Congenital ataxia, hypotonia, episodic breathing dysregulation and mental retardation	[101–103]
Rhombencephalosynapsis	Defective dorsal patterning and proliferation in the rhombic lips during early foetal development	Absence or severe dysgenesis of the cerebellar vermis. This leads to fusing of the two cerebellar hemispheres, peduncles and in the CN so that morphologically, there seems only to be one dentate nucleus that spans the breadth of the white matter	Cerebellar dysfunction, hypotonia, nystagmus, ataxia and mild to severe mental and motor developmental delays	[60, 104–106]

(continued)

Table 1 (continued)

Disorder	Aetiology	Pathology	Clinical features	Reference
Thanatophoric dysplasia	Due to gain of function mutations of FGF receptor 3 (FGFR3), which is involved in various parts of brain development, so pathological features are widespread across many brain regions as well as bones	Primarily a skeletal dysplasia with macrocephaly. Within the cerebellum, there are abnormalities of the cerebellar cortex, and CN are enlarged and hyperconvoluted and dysplastic. There is also dysplasia of the inferior olive	Generally is a lethal condition where foetuses are usually stillborn or die as neonates due to respiratory failure. For the very few survivors, clinical symptoms include seizures, dependence on ventilator and mental and motor impairments	[107–109]
Pontocerebellar hypoplasias	A group of neurodegenerative autosomal recessive disorders. Some variants are caused by tRNA splicing endonuclease mutations	Common feature is cerebellar hypoplasia and cerebellar and pons atrophy. In the cerebellum, there is scattered loss of PCs and segmental loss of dentate CN neurons, while specific regions of CN are preserved	Severe mental and motor impairments as well as swallowing problems and seizures	[110, 111]
Autism spectrum disorder	Heterogeneous: it may be caused by genetic, epigenetic or environmental factors during neurodevelopment. There is some consensus in that brain connectivity is affected. In the cerebellum, lower levels of GABA synthesis have been found in CN and PCs	Cerebellar vermal hypoplasia, reduction of superior cerebellar peduncle, decreased connectivity between the DN and cerebral regions (dentatorubrothalamic tract)	Heterogeneous spectrum or clinical features affecting social interaction, communication and behaviour	[112–114]

unanswered. A defining feature of development is that cells transit through the NTZ, yet nothing is known of the factors that regulate nucleogenesis.

Similarly, there are relatively few reports that highlight differences in cell types across the different CN. For example, Bagnall et al. [15] identified projections that are restricted to the fastigial CN, while molecular and cellular analyses point to underlying temporal cues that may explain how different nuclei are formed [67, 71]. Given that different densities of CN cell types are found across the already diversely shaped CN, and that the various CN have been found to be involved with wide ranges of motor control, from eye blinks to posture, it may be that connectivity and plasticity differ across similar cells to bring about an assortment of functions.

Finally, the diversity of different CN cells types, their origins and how they develop a network of intranuclear connectivity are key developmental questions whose answers will be of huge significance for functional models of the cerebellar network. The answer to these questions may also point towards new landmarks for the identification of disease processes in the cerebellum. This somewhat neglected population of brain cells is poised at a threshold of new understanding that offers the promise of new perspectives on the both how the cerebellum works and its clinical vulnerabilities.

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Motor Circuit Abnormalities During Cerebellar Development

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Abstract The cerebellum controls ongoing motor function and motor learning. Therefore, damage to its circuits causes a number of movement disorders such as ataxia, dystonia, and tremor. Cerebellar connectivity in both normal and abnormal states has been intensely studied. As a result, its anatomy, circuitry, and neuronal firing properties are among the best understood in the brain. This knowledge has directly facilitated efforts to uncover the mechanisms that cause motor dysfunction. Here, we discuss several mouse models of cerebellar disease. We focus on how cerebellar development depends on genes and neural activity to assemble circuits for behavior.

Keywords Cerebellum • Circuitry • Ataxia • Purkinje cell • Cerebellar nuclei • Inferior olive

Introduction

The cerebellum is best known for its crucial role in controlling smooth, purposeful movements. Cerebellar circuits receive motor planning information from the cerebral cortex about the goals and commands of movement in addition to feedback

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information from the brain stem and spinal cord about the sensory consequences of movement execution. This activity within the cerebellum can be modified through multiple cellular and molecular mechanisms of synaptic plasticity. The resultant output of cerebellar activity influences connected motor systems in the cerebral cortex, brain stem, and spinal cord to allow for calibration of motor programs that can be initiated and executed without immediate sensory feedback. There are currently two general models for how the cerebellum controls motor behavior during both ongoing movement (motor coordination) and repetitions of the same movement (motor learning). One model is that cerebellar computations evaluate the accuracy of actions by comparing predicted outcomes of intended movements to the outcomes of actual movements and then reduce error by providing signals for adaptive corrections [1]. The other model is that the cerebellum participates in the timing of movement rather than error correction [2]. It is also possible that the cerebellum performs both functions. In either case, it is not surprising that physical, pharmacological, and genetic insults to the cerebellar circuit result in movement disorders, and descriptions of motor symptoms after cerebellar damage date back to Flourens [3], Babinski [4–6], Holmes [7], and other pioneers in the field [8]. Cerebellar insults typically disrupt the coordination and accuracy of movement, conditions cumulatively referred to as "ataxia" (Greek, loss of order). Numerous distinct motor symptoms can arise from cerebellar damage, including the inability to judge distance or scale during target-oriented movements ("dysmetria," Greek, abnormal measure), oscillatory shaking of muscles during movement (tremor), diminished reflexive resistance to passive limb displacements ("hypotonia," Greek, low tone), and impaired production of speech ("dysarthria," Greek, abnormal articulation). Symptoms arise from the loss or disruption of normal cerebellar functions, and the ultimate motor behavioral consequences may also be due to movement control in a pathological state. Here, we discuss the mechanisms for different manifestations of cerebellar disease from the perspective of insights gained from mouse models, as they are currently one of the most common tools used in the study of cerebellar disorders. In order to understand the behavioral consequences of the diseased cerebellar circuit, we will consider cerebellar structure and development in the context of the functional motor system in vivo.

Structure of the Cerebellum

The cerebellum is interconnected with the rest of the brain by three pairs of large fiber tracts on its ventral surface, the cerebellar peduncles, and located dorsal to the pons and medulla (see chapter "The Embryology and Anatomy of the Cerebellum"). Though it is a predominantly continuous structure, there are three gross anatomical divisions of the cerebellum: a "wormlike" region along the midline called the vermis (Latin, worm), lateral regions that are relatively enlarged in humans called the hemispheres, and an intermediate region called the paravermis. The cerebellum comprises a three-layered cortex surrounding an inner core of white matter and

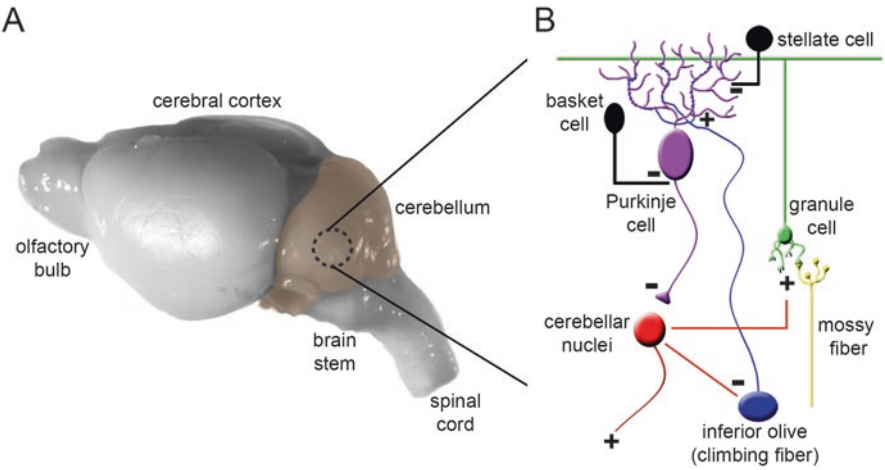

Fig. 1 Architecture of the cerebellar circuit. **(a)** Mouse brain shown from a lateral view with the cerebellum highlighted in color. **(b)** The basic cerebellar circuit comprises Purkinje cells, granule cells, stellate and basket cell interneurons, and the cerebellar nuclei. Afferent information is delivered to the cerebellum as climbing fibers or mossy fibers. Note that the Purkinje cell is the sole output of cerebellar cortex and the cerebellar nuclei deliver efferent information of the circuit. The + and – signs indicate whether each synapse is excitatory or inhibitory, respectively. For simplicity we have not shown Golgi cells, unipolar brush cells, Lugaro cells, or candelabrum cells. The different types of glia were also omitted. (Modified with permission from Reeber et al. [13])

three pairs of cerebellar nuclei. The sheet of cortex folds as cells proliferate during cerebellar development into folia and fissures along the anteroposterior axis, which form a series of lobules that are evolutionarily conserved and reproducible in mammals and birds [9]. Based on the work of Olof Larsell, Roman numerals are used to identify lobules in the vermis (I – X), whereas the hemispheres comprise CrusI, CrusII, lobulus simplex (LS), paramedian lobules (Pml), copula pyramids (Cop), the flocculus (Fl), and the paraflocculus (Pfl). Though lobule form is distinct across the anatomical divisions of the cerebellum, they contain the same repeated circuit and all the major cerebellar cell types [10–12] (Fig. 1), with the Purkinje cell at the center of each circuit. Purkinje cell somata form a monolayer, the Purkinje cell layer, across the cerebellar cortex and extend elaborate dendritic arbors into the outermost of the three layers, the molecular layer. Climbing fibers, one of the two major afferent pathways to the cerebellum, originate in the inferior olivary nucleus of the medulla and form excitatory synapses on the smooth shafts of Purkinje cell dendrites in the molecular layer. Mossy fibers, the second major afferent pathway to the cerebellum, terminate on granule cells within the third and innermost layer of cerebellar cortex, the granule cell layer, and originate from over two-dozen brainstem and spinal cord nuclei [14]. These nuclei include the basilar pontine nuclei relaying input from the cerebral cortex, dorsal nucleus of Clarke, vestibular nuclei, cuneate nuclei, and lateral reticular nuclei. Mossy fibers communicate with Purkinje cells indirectly through granule cell axons, known as parallel fibers, which ascend

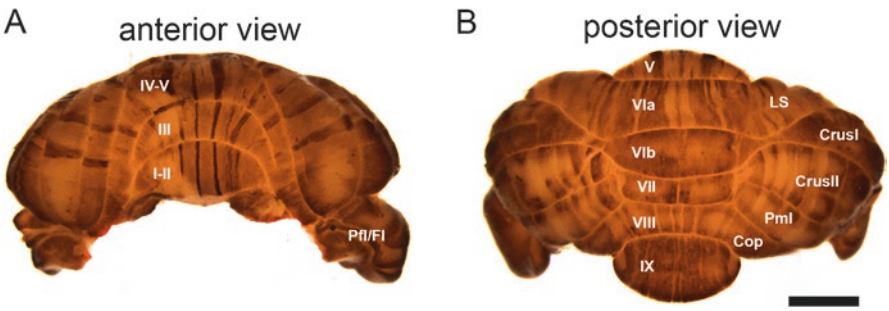

Fig. 2 ZebrinII zones in the mouse cerebellum. **(a**, **b)** Wholemount immunohistochemical staining of the mouse cerebellum with zebrinII reveals the intricate patterning of the cerebellar cortex into parasagittal zones. Roman numerals identify the lobules of the vermis. *Pfl* paraflocculus, *Fl* flocculus, *LS* lobulus simplex, *Pml* paramedian lobule, *Cop* copula pyramidis. Scale bar = 2 mm. (Modified with permission from Reeber et al. [13])

the granule cell and Purkinje cell layers and bifurcate to form excitatory synapses on the spines of Purkinje cell dendrites in the molecular layer. Numerous interneurons are present that influence the activity of local circuits, such as stellate and basket cells in the molecular layer and Golgi and unipolar brush cells in the granule cell layer. Neuromodulatory afferents also terminate in all three layers of the cerebellar cortex and within the cerebellar nuclei to influence local activity [15, 16]. Purkinje cell axons are the sole output of the cerebellar cortex and integrate all cerebellar inputs before projecting to the core of the cerebellum to form inhibitory synapses on their target cerebellar nuclei neurons. The cerebellar nuclei are the final efferent pathway to the rest of the brain and spinal cord; however, a small minority of Purkinje cells project directly to vestibular nuclei [17]. Despite this relatively simple and repeated cytoarchitecture (Fig. 1), a more complex circuit map is revealed by molecular, anatomical, and physiological approaches and by symptoms of disease. Subsets of Purkinje cells are divided into a series of reproducible parasagittal stripes, "zones," (Fig. 2) that run along the anteroposterior axis and are defined by gene expression patterns [12]. The classical and most thoroughly studied molecular marker of zones is known as zebrinII, which is an antigen on the metabolic enzyme aldolase C [18]. The topographical map of zebrinII expression in mice has been detailed extensively [19–21]. However, zebrinII is conserved, and its general pattern of expression is identical across different taxa [22–28]. ZebrinII-expressing Purkinje cells alternate with zones that do not express the antigen. Together, the two subsets form a striking array of zebrinII-positive and zebrinII-negative stripes that are symmetrically distributed across the midline. More than 40 molecular markers of zones have been identified [29] including excitatory amino acid transporter 4 (EAAT4), phospholipase C beta 3 (PLCβ3), and gamma-aminobutyric acid type B receptor subunit 2 (GABAβR2), which are expressed in zebrinII-positive zones, and phospholipase C beta 4 (PLCβ4), metabotropic glutamate receptor 1 splice variant 1b (mGlurR1b), and neuroplastin, which are expressed in the complementary zebrinII-negative zones. Bands of zones do not run

uninterrupted from anterior lobules to posterior lobules, and a unique pattern of zones is observed in four domains of the vermis: anterior = lobules I – V, central = lobules VI – VII, posterior = lobules VIII and dorsal IX, and nodular = lobules ventral IX and X [30] (Fig. 2). These domains are also innervated by functionally distinct mossy fiber afferents; for example, the spinocerebellar tract projects to the anterior and posterior domains, the pontocerebellar tract projects to the central and posterior domains, and the vestibulocerebellar tract projects to the nodular domain [12, 31]. These domains are not equivalent to the traditional functional compartments known as the spinocerebellum (regulation of muscles, tendons, and joints), cerebrocerebellum (planning and initiation of movement), and vestibulocerebellum (body equilibrium and oculomotor function). However, there is clearly some overlap in the functional attributes of each. These divisions are also reflected by the phenotypes of cerebellar disease in naturally occurring mutant mice, which often display differential structural defects along the anteroposterior axis [30]. Furthermore, the axon termination patterns of mossy and climbing fiber afferents within each of these domains exhibit parasagittal zones that have a reproducible anatomical relationship with the zones of their target Purkinje cells [32, 33] or the narrower functional microzones [34]. Climbing fibers originating from a specific subnucleus of the inferior olive typically terminate in one or two of these longitudinal zones [35, 36], and mossy fibers from specific sources branch to terminate in multiple longitudinal zones [37–40]. Zones are also distinct in their topographically defined Purkinje cell output to specific subnuclei of their three target cerebellar nuclei, fastigial (medial), interposed (middle; = globose and emboliform in primates), and dentate (lateral), each of which, too, has a unique efferent pathway to the rest of the brain and spinal cord [31, 41, 42], including projections back to the inferior olive to form a patterned cortico-nucleo-olivary tripartite loop [43, 44]. Together, units of topographically organized cerebellar afferents, their target Purkinje cell zones, and Purkinje cell efferent projections to the cerebellar nuclei compose cerebellar "modules," the basic functional circuit of the cerebellum [45]. Retrograde transsynaptic tracing shows that individual muscle groups are linked to specific Purkinje cell zones [46]. Functional mapping of the cerebellar circuit using imaging and electrophysiology also exhibits topography consistent with the zonal plan [47–50]. Within each zone, receptive fields mapped by recording responses to tactile stimuli reveal a "fractured somatotopy" of spinocerebellar mossy fibers with multiple sensory representations of body parts in mosaic patches [47, 51, 52]. Due to the relatively uniform cytoarchitecture of the cerebellum, it has been thought that these topographical differences in function are caused by differences in afferent and efferent connectivity; however, recent evidence suggests that this is also due to other regional variations such as Purkinje cell morphology, Purkinje cell packing density, granule cell packing density, neuronal soma size, the position of mossy fiber and climbing fiber synapses within their target layers, distribution of interneurons, intrinsic Purkinje cell firing properties, and synaptic plasticity [53]. Distinct computational processes within and between zones can potentially arise from variations in the cytoarchitecture and physiology of local circuits in these functional compartments. This exquisite organization of connections and the precise circuitry they

form require carefully executed developmental programs for proper function and behavior [54]. During this complex coordination, there are many opportunities for insults to cause disorders with devastating consequences for motor and perhaps even non-motor behavior.

Development of the Cerebellar Circuit

Due to the cerebellum's well-understood circuitry and roles in developmental and adult-onset diseases, it is an important model for understanding normal and abnormal brain circuit map formation [54]. Positional cues must be present to set up the patterns of specific lobules on the anteroposterior axis and zones on the mediolateral axis. Studies resolving how genes establish the coordinates of this functional framework have increased our understanding of the impact of complex neurological diseases [12]. The embryonic cerebellum is initially smooth without external morphological landmarks, but fissures that distinguish five cardinal lobes in the vermis begin to form by late embryonic development, embryonic day 17 (E17) in mice. Purkinje cells are derived from the ventricular zone of dorsal rhombomere 1 from E10 to E13 and migrate along radial glia into symmetrical clusters by ~E14. The granule cells are derived between ~E12 and E17 from a germinal zone called the rhombic lip, which produces a specialized transient progenitor layer on the surface of the cerebellum called the external granule cell layer by E16.5 [54]. Cerebellar granule cells are the most numerous cell type in the adult brain. They undergo extensive proliferation and are the main driving force for cerebellar growth and lobule patterning. During postnatal development, the five cardinal lobes expand and fold as they subdivide into the conserved stereotyped lobules, and this process (foliation) is complete by postnatal day 14 (P14) in mice. Genetic cues allowing for the precision and reproducibility of foliation between animals are not fully understood but may involve the "anchoring" of Purkinje cells to the future base of lobules by their projections to the cerebellar nuclei and the proliferation of granule cell precursors mechanically forcing lobule outgrowth [55] under the control of Purkinje cell-derived sonic hedgehog (Shh) signals [56, 57] and the function of *Engrailed* homeobox genes (*En1/2*) [58, 59]. The molecular heterogeneity of Purkinje cells may provide a scaffold that guides the patterns of neural circuit formation in the developing cerebellum, which is consistent with evidence that Purkinje cell subsets differentially express intrinsic molecular markers as early as E14 [60–62], including cell adhesion and guidance molecules [63, 64]. Purkinje cells are critical not only for shaping morphogenesis but also for guiding topographical map formation. Purkinje cells of similar birthdates may determine the adult patterns of Purkinje cell gene expression and restrict the boundaries of zones as the map forms. This is accomplished during embryogenesis when Purkinje cell subsets migrate and cluster into similar coordinate positions [65]. Afferents arrive in the cerebellum spanning mid-embryonic and postnatal development [66] in positions that later correspond to specific lobules, and Purkinje cell cues are thought to provide the scaffold that

guides afferents into longitudinal zones following the initial patterning of Purkinje cell clusters [54]. Retrograde tracing in fixed embryonic rat tissue shows that mossy fibers from the vestibular ganglion arrive in the cerebellum by E13, and those from the vestibular nuclei and spinal cord arrive at E15 [66]. Climbing fibers arrive at ~E17, followed by mossy fibers from the lateral reticular nucleus and pontine nuclei at P0 [66]. In mice, spinocerebellar and vestibular mossy fibers arrive at E13/14 [67], climbing fibers arrive at E14/15 [68], and the remaining mossy fibers arrive during late embryonic and postnatal development [54]. Climbing fiber afferents exhibit rudimentary parasagittal stripes by E15/16 in mice [68], soon after Purkinje cell clusters initially express transient parasagittal molecular markers such as *En1/2* [61]. Climbing fiber termination patterns and Purkinje cell zones correspond topographically by E17 [69]. Though mossy fibers synapse on granule cells in the adult cerebellum, they form transient contacts with Purkinje cells during embryonic development that may be critical for the segregation of spinocerebellar afferents into parasagittal zones [32, 70–73]. Unlike climbing fibers, mossy fibers do not exhibit clear-cut zones until after birth [74]. Purkinje cells are innervated by five to six climbing fibers by P3, and during early postnatal development one of these connections is selectively strengthened while the other synapses are eliminated; by P17 each Purkinje cell is innervated by a single climbing fiber, and each climbing fiber may contact up to ten Purkinje cells [75]. Cerebellar postnatal development also involves changes in the firing properties of both Purkinje cell simple spikes, which are intrinsically generated and modulated by mossy fiber to granule cell inputs via granule cell parallel fiber projections, and Purkinje cell complex spikes, which are generated by climbing fiber afferents [76] (Fig. 3). Both frequency and regularity of Purkinje cell spikes are dynamic as climbing and parallel fiber synapses mature and intrinsic Purkinje cell gene expression changes during development [76]. Neural activity, mediated by spontaneous activity and sensory experience, likely intersects with genetic programs to properly assemble the cerebellum and its circuits [77]. Genetic mouse models demonstrate that if genes regulating organization of the circuit are disrupted, there are severe impacts on map formation and motor function although external morphological defects typically associated with cerebellar disease may be subtle. For example, *En1/2* genes are critical for establishing the organization of the cerebellar circuit, and *En1/2* mutants exhibit altered formation of lobules and parasagittal Purkinje cell gene expression [59, 78–81]. Furthermore, adult patterns of mossy fiber afferents in distinct lobules and parasagittal zones are sensitive to *En1/2* deletions [72]. Spontaneous mutant mouse models of ataxia identified by their motor phenotypes also demonstrate an active role for Purkinje cells in setting up the topography of cerebellar afferents and the importance of the cerebellar circuit map for motor control. Mossy fiber termination patterns are altered in the *staggerer* mutant mouse with intrinsically affected Purkinje cells [70]. The *dreher* mutation causes cell fate changes of cerebellar progenitors, and anteroposterior and parasagittal patterns are distorted but present despite external morphological phenotypes [82]. The cerebellar deficient folia (*cdf*) mutation causes a selective failure of a zebrinII-positive Purkinje cell cluster to disperse, and adult mutants have abnormal parasagittal zone widths in the anterior vermis [83]. *Scrambler* mutant mice are

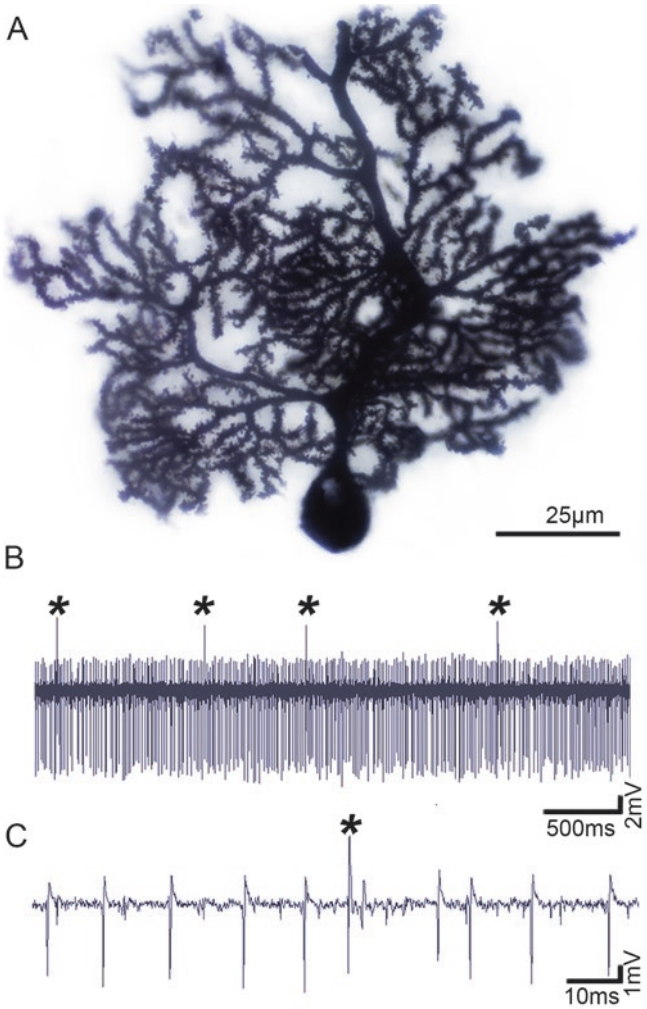

Fig. 3 Purkinje cells fire simple spikes and complex spikes. **(a)** Purkinje cell labeled using the classical Golgi-Cox staining method, demonstrating the elaborate morphology and dendritic branching of the Purkinje cell. **(b)** Extracellular single-unit recording from a Purkinje cell of an adult mouse in vivo. Purkinje cells fire two types of action potentials: high-frequency simple spikes that are driven by intrinsic activity and modulated by mossy fiber-granule cell inputs and low-frequency complex spikes that are triggered by climbing fiber input (*astericks*). **(c)** Higher power image of the Purkinje cell recording shown in panel (**b**) with individual spike waveforms visible. (Modified with permission from Reeber et al. [13])

able to maintain Purkinje cell zones and topographical circuits despite the abnormal placement of 95 % of Purkinje cells due to severe ectopia [84]. The *reeler* mutation causes the cerebellum to contain a "single lobule" composed of hypogranular cortex and a central mass of Purkinje cell clusters mixed with cerebellar nuclei, but the spinocerebellar and vestibulocerebellar afferents of *reeler* mice are able to maintain

targeting to specific regions despite the lack of external morphological landmarks [85, 86]. These mouse models of motor dysfunction, which have cerebellar abnormalities due to structural and circuit defects, have been invaluable for furthering our understanding of how circuit maps are generated. Moreover, the use of spontaneous and engineered (knockout and conditional) mice has helped shed light on the mechanisms of complex cerebellar diseases.

The Role of Cerebellar Development in Ataxia, a Classical Cerebellar Movement Disorder

As the genes and specific mutations causing human disorders continue to be identified, genetic mouse models of individual diseases have shed light on how the cerebellum is affected at the levels of pathology, physiology, and circuit patterning to cause symptoms with which patients present in the clinic. Ataxia is the most common symptom of cerebellar disease and a common phenotype of the aforementioned mutant mice. Upon neurological examination, patients with ataxia usually exhibit uncoordinated limbs, impaired balance, gait disturbance, and diminished fine motor control [87]. Cerebellar ataxia is the most common form of ataxia, and there currently are over 60 identified forms of inherited cerebellar ataxia [13, 88]. Although ataxia and other cerebellar motor deficits are typically discussed in relation to specific genetic mutations, defects in cerebellar circuitry can also be sporadic or acquired as a result of stroke, tumors, multiple sclerosis, alcoholism, peripheral neuropathy, metabolic disorders, and vitamin deficiencies [89]. The following genetic cerebellar manipulations demonstrate the diversity of paths that can lead to ataxia and related motor deficits. We focus on Purkinje cells due to their crucial role during cerebellar development and their central function in the adult circuit.

SCA1 (Spinocerebellar Ataxia Type 1)

Spinocerebellar ataxia type 1 (SCA1) is a dominantly inherited form of ataxia. SCA1 causes progressive loss of motor coordination, impaired balance, and gait disturbance. Other symptoms typically include dysarthria, dysmetria, difficulty swallowing, muscle atrophy, kyphosis, nystagmus, spasticity, and cognitive impairments [90]. SCA1 belongs to a family of neurodegenerative conditions that are caused by abnormal CAG repeat expansions that encode polyglutamine tracts. The mutated gene responsible for SCA1 was cloned and identified as the transcriptional regulator *ATAXIN-1* [91]. The polyglutamine ataxin-1 protein product is widely expressed in the brain but in SCA1 becomes toxic primarily to Purkinje cells of the cerebellum [92]. Polyglutamine ataxin-1 remains uniquely soluble in Purkinje cells, allowing it to enter the nucleus and disrupt the function of multiple protein complexes [93]. In humans, the onset of motor deficits most often occurs in the third or

fourth decade of life followed by death 10–15 years later; however, the age of onset and survival time depend on the number of repeats in the expanded polyglutamine sequence and can occur as late as the sixth decade of life or as early as the first decade [94]. Neuroimaging of late-stage SCA1 patients reveals gross atrophy of the cerebellum primarily due to the degeneration of Purkinje cells [90, 92, 95]. SCA1 patients also typically exhibit atrophy of the dentate cerebellar nuclei, pons, inferior olive, and other brain stem nuclei as the disease progresses [92]. Thus, degeneration eventually impacts both the cerebellar afferent and the efferent pathways. Postmortem examination of cerebellar tissue from SCA1 patients shows morphological abnormalities of remaining Purkinje cells in addition to Purkinje cell loss [95, 96]. The generation of mutant SCA1 transgenic mice has been critical in furthering our understanding of SCA1 progression [97–99]. First, electrophysiological properties of Purkinje cells such as intrinsic firing and the strength of glutamatergic synapses are abnormal preceding both onset of ataxia and Purkinje cell structural alterations in SCA1 mutant mice [100, 101]. Furthermore, specific genes involved in glutamate and calcium signaling are downregulated in Purkinje cells of SCA1 mutants before the morphological changes or behavioral deficits are obvious [102, 103]. Impaired performance on motor tasks in SCA1 mutant mice appears subsequently but before Purkinje cell morphological changes [100], suggesting changes in gene expression and altered circuit activity initiate SCA1 symptoms rather than the degeneration of Purkinje cells. Motor performance continues to decline as the dendritic morphology of Purkinje cells begins to deteriorate, dendritic arborization is reduced, the number of dendritic spines decreases, and the molecular layer shrinks as cells regress [97, 100]. Structural abnormalities become more evident as the proximal Purkinje cell dendrites atrophy and when the Purkinje cell somata begin to exhibit heterotopic positioning in the molecular layer [97, 99, 100]. It is not until the later stages of disease progression that Purkinje cell loss is detected [97, 99, 100]. The ages at which these events occur in SCA1 mutant mice differ between models containing shorter or longer knocked-in CAG repeats, consistent with what is observed in human patients [94]. The longer repeats cause an earlier onset of the disease and more severe symptoms. Despite the earlier onset, analysis of disease progression in juvenile and young adult mutant mice reveals that abnormalities in circuit activity and motor performance precede Purkinje cell degeneration. Progressive impairment of motor function in SCA1 thus reflects not only the degeneration of cells in the cerebellum and associated brain stem nuclei but also the earlier and sustained dysfunction of key neuronal populations that are integrated within the circuit.

SCA6 (Spinocerebellar Ataxia Type 6)

Spinocerebellar ataxia type 6 (SCA6), like SCA1, is a dominantly inherited form of ataxia and a triplet repeat disease. In SCA6, a CAG repeat expansion occurs within the gene *CACNA1A*, which encodes the pore-forming subunit of voltage-dependent

P/Q-type calcium channels [104, 105]. The mutated polyglutamine P/Q-type calcium channels are widely expressed in the brain but become toxic primarily to Purkinje cells [106], where they are highly expressed in the plasma membrane [107]. Age of onset and survival time depend on the number of repeats in the expanded polyglutamine sequence, but SCA6 onset most commonly occurs in the fifth or sixth decade of life followed by death 20–30 years later [94]. SCA6 patients experience slowly progressive ataxia of the limbs and gait in addition to dysarthria and nystagmus [104, 108], and neuroimaging reveals cerebellar atrophy [108]. Neurodegeneration in SCA6 occurs mostly in Purkinje cells, but death of neurons in the dentate cerebellar nuclei and inferior olive is also observed [105, 109, 110]. Postmortem examination of cerebellar tissue from SCA6 patients shows morphological abnormalities of remaining Purkinje cells in addition to the loss of Purkinje cells [106]. In transgenic mouse models of SCA6, the onset of ataxia occurs before morphological changes or loss of Purkinje cells [111]. Electrophysiological examination reveals that Purkinje cells exhibit reduced firing rates and rhythmicity at ages coinciding with the onset of ataxia [112] and at later disease stages [113]. Though the polyglutamine mutation occurs in an ion channel that regulates the firing patterns of Purkinje cells in adult mice [114], SCA6 symptoms do not result from changes in channel current but rather age-dependent gain-of-function effects of aggregated mutant protein on cellular function [113, 115, 116]. Although SCA6 symptoms manifest in midlife, P/Q channels are expressed soon after birth [117] and are involved in synapse elimination of climbing fiber innervation onto Purkinje cells during development [75, 118, 119]. Interestingly, Purkinje cells of SCA6 mutant mice exhibit transiently increased firing rates and rhythmicity as well as abnormal climbing fiber innervation during early postnatal development without causing behavioral abnormalities [120]. These alterations disappear once the mice reach weanling age when the circuit has largely developed [54], and cellular and synaptic function of Purkinje cells return to normal [120]. These transient electrophysiological phenotypes during development are different from those observed in adult SCA6 mice, and they do not appear to impact motor coordination nor represent a mild initial stage of the ultimate phenotype that would progressively worsen. However, compensatory adaptations prior to disease onset have been observed in the Purkinje cells of SCA1 mutant mice [101], and such homeostatic alterations to the cerebellar circuit in response to transient electrophysiological dysfunction have not yet been detected in developing SCA6 mice but may not become pathological until later in life, if they are present [120]. In addition to SCA1 and SCA6, a prolonged period of Purkinje cell dysfunction prior to neuronal loss has emerged as a common feature in other polyglutamine disorders including spinocerebellar ataxia type 3 (SCA3); Purkinje cells in a genetic mouse model of SCA3 exhibit abnormal intrinsic activity and motor symptoms prior to neurodegeneration [121]. These early manifestations of hereditary ataxias could be effective targets for therapy as the circuits could be rescued before the cells die [100, 112, 121].

Car8^{wdl} (The waddles Spontaneous Mutant Mouse)

The carbonic anhydrase 8 gene (*Car8*) is abundantly expressed in Purkinje cells [122, 123]. Lower levels of expression can also be seen in the cerebellar nuclei and brainstem due to the termination of Purkinje cell axons in these regions. The CAR8 protein is involved in calcium modulation pathways [124] and is expressed beginning during embryonic development and continuing into adulthood [125, 126]. A spontaneous mutant mouse, *waddles* (*Car8^{wdl}*), contains a deletion within the *Car8* gene and exhibits progressive ataxia that is evident by 2 weeks of age in addition to appendicular dystonia and tremor [122]. In humans, mutations in the homologous gene (*CA8*) also cause ataxia [127]. Unlike in the SCAs, Purkinje cells do not degenerate, and the cerebellum does not show gross anatomical defects [122, 123]. However, adult *Car8^{wdl}* mice have microcircuit abnormalities including denser climbing fiber innervation that extends to distal Purkinje cell dendrites and reduced parallel fiber synapse formation on Purkinje cell dendritic spines [128]. The mutation also impairs the topography of cerebellar circuits during development; the segregation of Purkinje cell subsets into distinct parasagittal zones is developmentally delayed in *Car8^{wdl}* mice, and the topography of spinocerebellar afferents is abnormal in early postnatal and adult mice [123] (Fig. 4). Furthermore, electrophysiological examination of mutant mice reveals that the developing Purkinje cells exhibit abnormal firing frequency and patterns [123, 128], but Purkinje cells do not degenerate even as ataxia worsens [123]. The ataxia observed in *Car8^{wdl}* mice thus may result from both miswiring of the cerebellum's functional map and aberrant electrophysiological output of adult Purkinje cells. The CAR8 protein is a binding partner for inositol triphosphate receptor type 1 (IP3R1) [122, 124], an intracellular calcium release channel that is mutated in SCA15. Interestingly, *IP3R1* is one of the genes downregulated in SCA1 mice preceding the onset of ataxia or morphological changes [102, 103]. Impaired calcium homeostasis in Purkinje cells appears to mediate a central mechanism of pathogenesis common to many types of ataxia that manifest with or without neurodegeneration. However, CAR8 likely has calcium-independent functions as well.

*L7^{Cre};*Vgat$^{flox/flox}$ *(Conditional Genetic Silencing of Purkinje Cell Neurotransmission)*

Effective cerebellar control of motor behavior depends on the ability of Purkinje cells to integrate incoming sensorimotor inputs and communicate appropriately with their target neurons in the cerebellar nuclei. In the *L7^{Cre};Vgat$^{flox/flox}$* mouse, inhibitory synaptic transmission of Purkinje cells is constitutively blocked using conditional genetics [129]. Under control of the cell type-specific promoter *L7*, Cre recombinase excises the *floxed* vesicular GABA transporter gene (*Vgat*) that encodes the transporter for loading neurotransmitter into synaptic vesicles [129]. This eliminates the ability of Purkinje cells, the sole output of the cerebellar cortex,

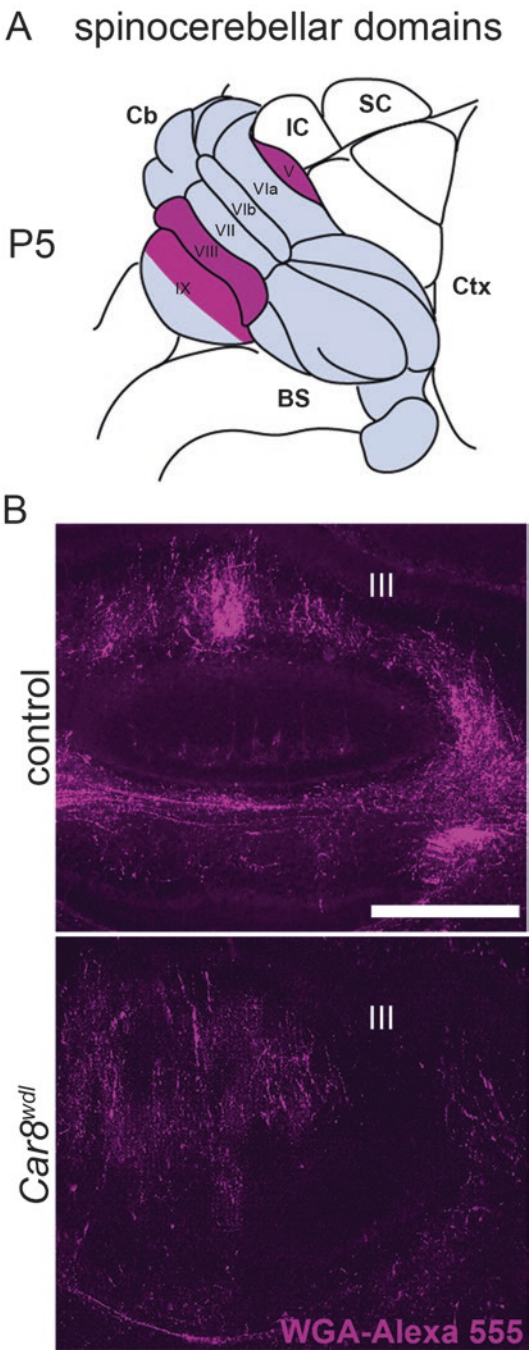

Fig. 4 The termination pattern of spinocerebellar mossy fibers is altered in *Car8*^{wdl} mice. **(a)** Schematic of the postnatal day 5 (P5) mouse cerebellum from a lateral view with the cerebellum highlighted in *blue* and the primary target domains of spinocerebellar mossy fiber projections highlighted in *magenta*. Roman numerals identify the lobules of the vermis. Note that the anteriormost lobules are also innervated by the spinocerebellar tract and are not visible as they are hidden from view by the colliculi. *Cb* cerebellum, *BS* brain stem, *Ctx* cerebral cortex, *IC* inferior colliculus, *SC* superior colliculus. (**b**) Fluorescent mapping of spinocerebellar mossy fiber terminal fields in lobule III of a *Car8*^{wdl} mouse and a control mouse at P5 after injection of WGA-Alexa 555 into the lower thoracic-upper lumbar spinal cord and transport of the tracer up the spinocerebellar tract. Mossy fiber topography is altered in *Car8*^{wdl} mice because the sensory pathways are incorrectly targeted and weakly innervate the cerebellum during early postnatal development. Scale bar = 250 μm. Panel **(b)** was modified with permission from White et al. [123]

to communicate with the cerebellar nuclei, the final output of the cerebellum and its link to the rest of the motor system. Purkinje cell output to the vestibular nuclei is also silenced by this approach. L7^{Cre};*Vgat*$^{flox/flox}$ mice exhibit motor coordination defects, gait disturbance, and impaired balance. Though the absence of Purkinje cell output does not affect the gross morphology of the cerebellum, segregation of Purkinje cells into zones is disrupted, and the zonal topography of spinocerebellar afferents develops abnormally [129]. Although the basic circuit map is intact, the normally sharp boundaries of zones are compromised [129]. Purkinje cells of *L7*^{Cre}*;Vgat*$^{flox/flox}$ mice exhibit abnormal electrophysiological activity, but their output is not signaled downstream in this model [129]. However, loss of Purkinje cell signaling causes the cerebellar nuclei to fire abnormally, impacting the ultimate output of the cerebellum. Taken together with other models of cerebellar dysfunction, it is clear that ataxia and other motor deficits can arise due to insults in wiring, firing, or survival of Purkinje cells in a wide range of diseases with diverse causes.

Cerebellar Development and Non-motor Disorders

Over the past 30 years, evidence from functional neuroimaging studies has mounted indicating that the cerebellum is active during non-motor behaviors such as perception, cognition, and emotion [130–132]. This idea is supported by evidence of extensive afferents and efferents interconnecting the cerebellum with prefrontal and parietal cortex [41, 133, 134]. Lesioning studies also suggest that cerebellar damage can lead to a variety of non-motor behavioral deficits [132, 135, 136]. However, the extent of the cerebellum's role in cognitive function remains unclear and is a topic of lively debate [137–140]. The adult cerebellum appears to be particularly relevant to those non-motor tasks requiring complex spatial and temporal judgments, such as prediction and perceptual sensory discrimination, or in which skilled mental responses are developed using an internal model [134, 141, 162]. It could be that the computational capacities of the cerebellum to discriminate patterns and use these patterns to learn to make context-dependent predictions with respect to motor behavior would be also useful to non-motor areas of the brain [142]. Signals from cerebellar cortex to both motor and non-motor areas of the cerebral cortex synapse in the interposed and dentate cerebellar nuclei and are then relayed through the thalamus [54]. In return, mossy fibers originating in the basal pontine nuclei relay information from cerebral cortex to the cerebellar cortex, with non-motor information likely going to the hemispheres [54]. Together, these cerebro-cerebellar connections form closed loops in which regions of the cerebellar cortex projecting to a given area of the cerebral cortex in turn receive input originating in those same areas of the cerebral cortex [41]. Each of these regions is involved in specific functions, forming a topographical map across the cerebellar cortex, cerebellar nuclei, thalamus, pons, and cerebral cortex [31, 41, 42]. Functional neuroimaging links different cognitive and motor behaviors to activity in specific cerebro-cerebellar closed loops [143], and focal cerebellar damage can cause different motor or non-motor deficits

in a location-dependent manner [132, 136]. This anatomical and functional segregation of cerebro-cerebellar connections might respect the modular architecture of the cerebellum [45]. Anatomical and functional abnormalities in the cerebellar circuit have been implicated in several non-motor neurodevelopmental disorders [144] and may play a particularly important role during sensitive periods of development [145]. Clinical studies have also noted increased cognitive deficits in children who suffer cerebellar damage during posterior fossa tumor resection [146]. How the cerebellum interacts with the cerebral cortex during development remains poorly understood. Some non-motor diseases linked to cerebellar development include autism spectrum disorder [145, 147, 148] and dyslexia [149, 150]. The cerebellum could also be involved in schizophrenia [151, 152]. The study of cerebellar non-motor diseases has required both human patients and genetic mouse models. For example, the most consistently affected structure in postmortem examination of tissue from autistic individuals is the cerebellum, including hypoplasia and reduced numbers of Purkinje cells without signs of neurodegeneration [147, 153, 154]. The *En2* gene is necessary for establishing the structure and circuit organization of the cerebellum during mouse development [54], and *EN2* mutations are linked to autism susceptibility in humans [155–157]. Loss-of-function mutations and transgenic misexpression of *En2* in mice cause autism-like behaviors [158, 159]. These mice show some morphological abnormalities in the cerebellum that are broadly similar to those reported in humans with autism as well as abnormal foliation and afferent topography [59, 79–81]. In addition to cerebellar defects being implicated in non-motor diseases, cerebellar "motor" diseases can also feature non-motor symptoms. For example, human and mouse studies show that SCA1 [99, 160] and human *CA8* mutations [127] cause cognitive deficits in addition to ataxia. It could be that the Purkinje cell and its associated microcircuits underlie both motor [129] and non-motor problems [162]. This would suggest that the basic operational properties of a Purkinje cell could be tuned to different behaviors. Future experimental work will reveal whether this is the case.

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Developmental Disorders of the Cerebellum and Neurotrophic Factors

Leila Pirmoradi, Ali Akbar Owji, and Shahla Shojaei

Abstract The cerebellum plays a main role in motor control and also in cognition features such as attention. Thus, a disturbance in cerebellar development results in neurological disorders such as attention deficit hyperactivity disorder (ADHD), congenital ataxia, and autism. Because neurotrophic factors have established effects on the growth, proliferation, differentiation, and arborization of neurons, their role in the neurodevelopmental disorders has been investigated for decades. Results of numerous studies have shown changes in serum or tissue neurotrophic factor levels, as well as alterations in their receptors and components of their signaling pathways in these types of the neurodevelopmental diseases. In this chapter, we provide a brief overview of neurotrophic factors and their role in cerebellar development and then focus on the roles of the neurotrophin system in developmental disorders and diseases of the cerebellum.

Keywords Cerebellar disorders • Attention deficit hyperactivity disorder • Congenital ataxia • Nerve growth factor • Neurotrophins • Transforming growth factor beta • p75 neurotrophin receptor

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Introduction

The cerebellum coordinates motor function and preserves equilibrium [1, 2], and it is also an important region of the brain for behavior and cognition in all aspects including language, memory, sleep, attention, and spatial and social emotional processing [2–4]. Early damage to the cerebellum results in more drastic and long-lasting effects on movement and cognition [5]. Early abnormalities in cerebellar function and regulation result in developmental disorders such as autism, ADHD, developmental dyslexia, and Joubert syndrome [5–7]. Many studies have investigated the molecular mechanism of cerebellar development, and the role of neurotrophic factors is well known [8, 9]. In the cerebellum, neurotrophic factors have a crucial effect on the generation, differentiation, and proliferation of different types of neuronal cells such as granule cells, Purkinje cells, and glia [8, 9], and dysregulation of their pathways was associated with developmental disorders in the cerebellum [10–12].

Neurotrophic Factors

Neurons and glial cells are dependent on growth factors for their normal function, differentiation, and survival [13]. The neurotrophin family of peptides was the first discovered family of growth factors that affect the central nervous system [14]. Neurotrophic factors modulate formation of the central nervous system by affecting the development and differentiation of neuronal cells in utero [14]. These proteins are also expressed throughout life and have central roles in the regulation of action and survival of neurons and glial cells [15, 16]. Receptors for these factors have also been discovered in many tissues where they mediate a wide range of actions including the morphogenesis of kidney and differentiation of vessels and immune cells [17–19]. Neurotrophic factors are classified into three groups: neurotrophins (NTs), the transforming growth factor-beta (TGF-β) superfamily, and neurotrophic cytokines (Fig. 1) [20].

Neurotrophins

Neurotrophins (NTs) are the best-studied neurotrophic factors, and their concentration changes play a main and pivotal physiological role in neuron removal during nervous system development. In the adult, NTs protect specific populations of neurons in the CNS. They play a critical role in learning and memory and regeneration processes by facilitating synaptic transmission and plasticity. The NT family was first introduced by discovery of nerve growth factor (NGF). Brain-derived

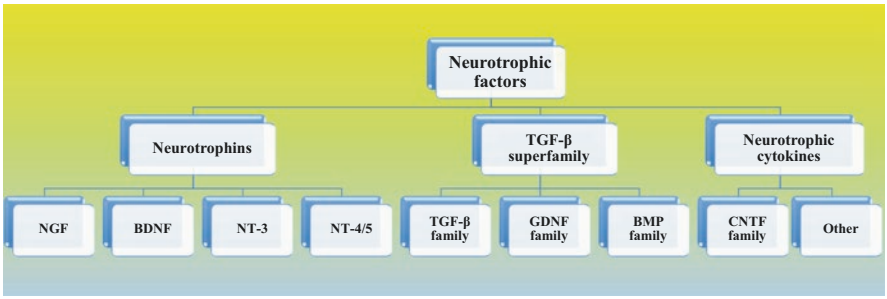

Fig. 1 Neurotrophic factors are classified into three main groups: (1) neurotrophins, (2) transforming growth factor-beta (TGF-β) superfamily, and (3) neurotrophic cytokines. Each of these groups is divided to their subgroup. *NGF* nerve growth factor, *BDNF* brain-derived neurotrophic factor, *NT-3* neurotrophin-3 and *NT-4/5* neurotrophin 4/5, *GDNF* glial-derived neurotrophic factor, *BMP* bone morphogenetic proteins, *CNTF* ciliary neurotrophic factor

neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin 4/5 (NT-4/5), also called neurotrophin-4 (NT-4) or neurotrophin-5 (NT-5), are other members of this group in mammals (Fig. 1) [20]. All NTs are synthesized in the form of precursor proteins and activated upon cleavage by metalloproteinases. They have two types of receptors: tropomyosin receptor kinase (Trk) from the tyrosine kinase family, which binds with high affinity, and p75 neurotrophin receptors (p75NTR) from the tumor necrosis factor (TNF) receptor superfamily, which has a low affinity for NTs. Each NT preferentially binds to its respective Trk receptor, resulting in Trk dimerization and subsequent tyrosine autophosphorylation, which in turn activates intracellular signaling pathways. NGF binds to TrkA, while BDNF and NT4 bind to TrkB, and NT3 binds to TrkC. Although pro-NTs cannot activate Trk receptors, they activate p75NTR to promote cell apoptosis via Rac1/c-Jun N-terminal kinase (JNK) pathways (Figs. 2 and 3) [20, 21]. Additionally, p75NTR can form a heterodimer with Trk receptors and lowering their affinity and promote survival through the nuclear factor kappa light-chain enhancer of activated B-cell (NF-κB) pathway. Disruption of the p75NTR signaling pathway has been observed in several autoimmune diseases [20].

Neurotrophins could exert a diverse effect following interaction with their cognate Trk receptors. They can increase neurotransmitter release through activation of the phospholipase Cγ (PLCγ) pathway, enhance synaptic delivery by activation of Ca^{2+}-/calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC). BDNF stimulates dendritic growth and spine maturation via interaction with TrkB. The actin cytoskeleton that has an important role in CNS function can be modulated by Trk signaling through activation of small Rho GTPases. Trk signaling also improves mRNA translation globally by induction of the phosphoinositide 3-kinase (PI3K)–AKT pathway and transcription of activity-regulated genes such as FOS and ARC [21].

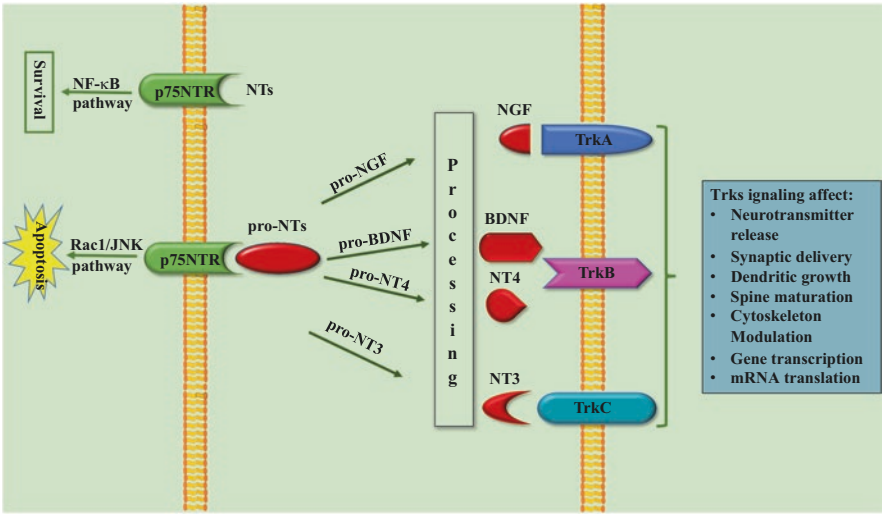

Fig. 2 Pro-neurotrophins (pro-NTs) activate p75NTR to promote cell apoptosis via Rac1/c-Jun N-terminal kinases (JNK) pathways. Upon processing to their cognate mature NTs, they interact to their specific Trks. NGF binds to TrkA, BDNF and NT4 bind to TrkB, and NT3 binds to TrkC. NTs can also interact with p75NTR with lower affinity and promote survival through nuclear factor kappa light-chain enhancer of activated B-cell (NF-κB) pathway. TRK signaling exerts diverse effect on the nervous system

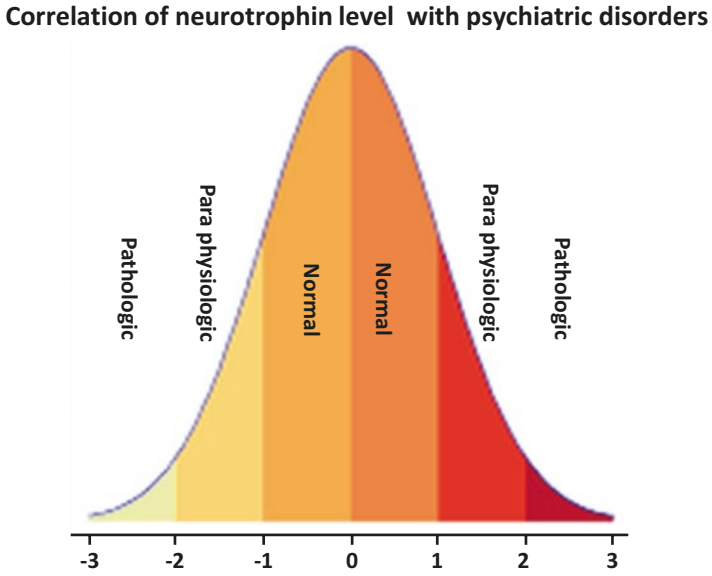

Fig. 3 Level of neurotrophins in the blood and/or neural tissues has been proposed as a determinant of vulnerability to psychiatric disorders

Transforming Growth Factor-Beta Superfamily

The TGF-β superfamily is a growing group with ubiquitous expression throughout the body and numerous roles in the growth and development of many organs. Members of this superfamily play a variety of roles such as control of the cell cycle, effects on differentiation, regulation of early development, formation of the extracellular matrix, modulation of hematogenesis, and immune reactions. The TGF-β superfamily comprises approximately 30 proteins in mammals that are divided into three families: TGF-β family, glial-derived neurotrophic factor (GDNF) family, and bone morphogenetic protein (BMP) family that each is subdivided to their individual members (Fig. 1) [20, 22]. TGF-β has three isoforms (1, 2, and 3) that have both protective and damaging effects on neurons based on the context of growth factors, cell type, and the developmental period [23–25]. GDNF was the first protein isolated in the GDNF family, and it has the most impact on the cerebellar neurons. GDNF protects dopaminergic, noradrenergic, and motor neurons in the midbrain and spinal cord, and it affects peripheral neuron morphogenesis [26]. In the cerebellum, GDNF assists with Purkinje cell function and survival [27, 28]. On the cerebellar granule neurons, GDNF has a protective effect against TGF-β cytotoxicity [24].

Neurotrophic Cytokines

Neurotrophic cytokines are a group of neurotrophic factors that are divided into two groups: the ciliary neurotrophic factor (CNTF) family and others (Fig. 1). CNTF was the initial protein discovered in this family, and it is a pluripotent neurotrophic factor. In the nervous system, CNTF affects survival and differentiation of sensory, sympathetic, and motor neurons and thereby influences development and maintenance of the nervous system [29].

Neurotrophic Factors and Cerebellar Development

NTs are present in the human cerebellum from perinatal age to adulthood, and their role in cerebellar connectivity has been confirmed [30]. BDNF and NGF are highly expressed in the cerebellum and cerebrum, and they have trophic effects in these areas. During development and in adulthood, growth factors including BDNF and NGF help neuronal plasticity in an activity-dependent manner and improve learning and memory [31]. NGF receptor expression in Purkinje cells shows the importance of NGF in the cerebellar development [32, 33]. Increased granule cell precursor proliferation and migration are characteristic features of postnatal cerebellar cortex development [34]. Purkinje cells are the target of other NTs like NT-3, but Tojo et al. showed that the deletion of this NT had no significant effect on the histological

characteristics of these cells [35]. Other studies discussed the survival effect of NT-3, in addition to NT-4 and BDNF [9]. Cerebellar Purkinje cells deprived of NTs die via a different form of apoptotic death, which occurs in adjacent granule cells, and this occurs because of excessive autophagy that is normally inhibited by NTs. P75NTR is necessary for Purkinje cell survival in the presence of trophic factors. P75NTR in the absence of neurotrophins induces Purkinje cell autophagy, and it is likely a mechanism that is involved in neurodegenerative diseases [36]. CNTF has a similar effect on Purkinje cell survival [9]. There was some controversy about the survival effect of BDNF in these cells and more specifically on the effect of this factor on their dendritic development discussed by Kapfhammer et al. [9]. They showed no significant effect of BDNF in the survival and dendritic development of Purkinje cells in the cerebellum. Neural proliferation during the developmental period and neural plasticity after brain injury both change the levels of the NFG in the cerebellum.

Studies on the role of NTs in neuronal survival and phenotypic differentiation at embryonic day 16 (E16) of the rat cerebellum showed that NGF failed to increase the number of Purkinje cells and GABAergic interneurons in cultures [37]. Instead, Kapfhammer et al. showed the survival-promoting effect of BDNF in these types of cerebellar cells [9]. Environmental enrichment (EE) partially affects on the cerebellum via upregulation of neurotrophins NGF and BDNF. Angelucci et al. showed that rats exposed to EE from weaning to 5 months of age showed a remarkable increase of both BDNF and NGF concentrations in the cerebellum compared with rats nurtured under standard conditions. This result shows the influence of EE on the cerebellum via NTs [38]. EE improves motor function after cerebellar damage in rats, which is attributed to regeneration processes caused by NTs [39]. The assessment of rats exposed to a microgravity environment in space for 3 months revealed no alteration in NGF expression in the cerebellum, while NGF expression in the hippocampus and cortex in the experimental group was less than rats in the ground control group [40]. Conversely, in neonatal rats exposed to hypergravity, there was a significant decrease of NGF expression in the cerebellum of neonates during birth on the postnatal day. However, the basic mechanisms by which NT acts in this condition are not yet known [41].

Biochemical pathways such as Notch, Wnt/β-catenin, TGF-β/BMP, Shh/Patched, and Hippo have critical roles in embryonic development. Among them, the TGF-β/BMP pathway has been shown to be most important in cerebellar development. Mutation and dysregulation of this pathway accompanied medulloblastoma, a type of CNS tumor originating from the cerebellum [42, 43].

Cerebellum and Neurodevelopmental Disorders

The role of the cerebellum is more than just motor activity. Because of the widespread connections between the cerebellum and other brain areas, the cerebellum has been considered to be a part of the brain that has a main role in emotion, cognition, behavior, and social interactions [3]. Thus, any damage to the cerebellum early

in development could have a deep impact on movement, cognition, and learning. Autism, ADHD, and developmental dyslexia are well-known developmental disorders of the cerebellum [5].

Attention Deficit Hyperactivity Disorder

Dysfunction of the cerebellum is a characteristic of some developmental disorders such as ADHD [5]. Studies implicate frontostriatal and frontocerebellar catecholaminergic circuit disorders in ADH pathophysiology [44] . Because antidepressants and psychostimulants used to treat patients with ADHD increase BDNF levels, it proposed that this neurotrophic factor plays an important role in the pathogenesis of ADHD [45]. Many studies on the pathogenesis of ADHD have focused on and confirmed the genetic association of the BDNF gene [46] or its polymorphisms [47–50] with ADHD. A recent large-scale DNA sequencing study supported this association [51]. The BDNF Val66Met polymorphism has been studied the most, but its association with ADHD is questionable. Park et al. showed a significant interaction between the neurotic symptoms of ADHA and the BDNF met allele in a Korean population [52]. However, a meta-analysis conducted on four European populations refuted the involvement of BDNF Val66Met polymorphism with pathogenesis of ADHD [53]. Recently, another study was performed to address this controversy [54].

Other investigators focused on the levels of neurotrophic factors in the blood, especially BDNF and its role in the pathogenesis of ADHD. The plasma level of BDNF in 41 drug-naive child ADHD patients was higher than that in 107 healthy controls [55]. A later study by the same group confirmed these findings [56], while Scassellati et al. showed no difference in the serum BDNF level between healthy and affected groups using the same samples [57]. A study enrolling Caucasian adult ADHD patients showed that these patients had decreased serum NT levels compared with the control group [58].

The role of NGF and its receptor (NGFR) has been shown in ADHD [44]. NGF exerts a trophic and functional role in the basal forebrain cholinergic neurons, which are involved in attention [59, 60]. Serum NGF levels were higher in drug-naive ADHD patients at childhood [61]. Bilgic et al. showed that serum NGF and BDNF levels in Turkish children were not significantly associated with ADHD, while serum GDNF and NT3 were higher in the patient group; however, they suggested that the NT serum level was not associated with severity of ADHD [26].

Autism Spectrum Disorders

Autism spectrum disorders (ASDs) are neurodevelopmental disorders that impairs communication and social ability. Both genetic [62, 63] and environmental [64] factors are involved in etiology of ASD, and cerebellar involvement in ASD has

been recognized [65, 66] (see chapter "Neurodevelopmental Disorders of the Cerebellum: Autism Spectrum Disorder").

In some animal models of ASD including Borna disease virus infection and rats treated with valproic acid, a gradual loss of Purkinje cells diminishes cerebellum size and induces other aspects of cognitive deficits [67]. Measurement of neurotrophin mRNA levels such as NGF, BDNF, and NT-3 and their respective TRK receptors in newborn rats infected with Borna disease virus showed that there were no alterations in the cerebellum. However, there were an increased number of apoptotic cells in the cerebellar granular layer and loss of cerebellar Purkinje cells [68]. A study on blood spot from newborns who were later diagnosed with ASD showed decreased NT-3 and NT4/5 levels compared with healthy subjects [69]. Similarly, in a postmortem study, the cerebellar NT-3 level was higher in ASD patient than in normal controls [10]. Another neurodevelopmental rodent model that mimics prenatal immune activation as an environmental risk factors for ASD and schizophrenia is the maternal lipopolysaccharide (LPS) exposure rat model [70]. LPS-treated pups on P21 show increased levels of cerebellar NT-3 [67].

Levels of neurotrophins are increased in the blood of children with ASD [69, 71]. The elevated levels of the serum NGF and other neurotrophins can be associated with the development of ASD and mental retardation later in childhood [72]. A variant type of BDNF has been found in autistic families in addition to increased blood levels of this neurotrophin in ASD children. Therefore, BDNF has been proposed as a critical factor that is involved in ASD and is a therapeutic targeted that is being studied [Reviewed in [73]]. Conversely, another review proposed a decreased blood level of BDNF as a marker for ASD prediction and prognosis [Reviewed in [74]]. Sadakata et al. reported that transgenic knockout mice that are missing Ca^{2+}-dependent activator protein for secretion 2 (CAPS2), a protein that is involved in NT release, were susceptible to autistic features [12, 75]. Nickl-Jockschat et al. discussed that altered neurotrophin levels are a pathological mechanism. As mentioned earlier, there is more affinity of pro-NT to activate p75NTR and subsequently more apoptotic cell death, and therefore the changes in the ratio of pro-NT to NT can result in some pathological aspects [76].

Neurotrophins such as NGF and BDNF play a role in dendritic morphology [77]. Dendritic shape abnormalities and a larger amount of dendritic spines have been detected in ASD patients. The cerebellum and inferior olive size variations have been reported in postmortem examinations of brains from ASD patients. These anomalies in dendritic branching happened in other neurodevelopmental disorders linked to ASD, such as Fragile X and Rett syndrome (RTT) [78]. RTT is a genetic disorder that is considered to be an ASD [79] (see chapter "Epigenetics and Cerebellar Neurodevelopmental Disorders"). However, for years there was a debate on the classification of RTT as an autistic developmental disorder, and in 2013, the American Society of Psychiatry changed the classification of RTT and removed it from the ASDs because of its unique molecular basis [80].

RTT affects girls, and mutations in the X-linked gene encoding methyl–CpG-binding protein 2 (MeCP2) are responsible for over 80% of affected girls [81, 82]. MeCP2 alters the expression of many genes in the cerebellum [83]. While serum

BDNF levels in RTT girls and in a normal group are similar, BDNF protein levels are reduced in RTT brains [79]. Reduced or unaltered NGF in the cerebellum and other brain regions has been reported [31]. Calamandri et al. showed that serum NGF levels decreased with age [84].

Ataxia

Cerebellar ataxias are neurological disorders that can affect vermis, paravermis, and hemisphere of the cerebellum during development [85] (see chapter "Motor Circuit Abnormalities During Cerebellar Development"). Machado–Joseph disease (MJD) or spinocerebellar ataxia type 3 (SCA3) is a hereditary ataxia that is caused by repeated CAG in the ATXN3 gene [86]. Neuronal loss in the cerebellar nuclei and Purkinje cell layer has been reported in MJD [86]. Since p75NTR has an important role in the induction of neuronal apoptosis, these findings encouraged researchers to investigate the role of p75NTR in the naked-ataxia mutant mouse, but p75NTR expression showed a normal pattern in this type of ataxia [87]. Because NGF and its receptor TkrA exist in human cerebellar neurons and are involved in cerebellar development and stability of the cerebellar connections, NGF therapy may improve symptoms in patients with SCA3 [88]. Jones et al. showed that mesenchymal stem cells improve survival of Purkinje cells by expression of BDNF, NT-3, or GDNF [89]. Detection of neurotrophin mRNA expression in the ataxic stargazer (stg) mutant mouse showed that NT-3 or NGF mRNA expression in the cerebellum was normal while BDNF mRNA in the cerebellar granule cell layer was reduced [90] . In the SCA6, decreased BDNF mRNA expression and altered BDNF protein levels in Purkinje cell dendrites have been shown [91] .

Ethanol Neurotoxicity

Ethanol exposure is a condition in which cerebellar cells are vulnerable to ethanol's neurotoxic effects during development (see chapter "Teratogenic Influences on Cerebellar Development"). In addition to damage to different parts of the brain including ventricular enlargement, cortical white matter shrinkage, and hippocampal abnormalities, the brains of alcoholics have shown a significant cell loss [92, 93] and white matter degeneration in the cerebellum [92] . Duc et al. reported that prenatal granular neurons exposed to ethanol in vitro are more sensitive to hypoxic/hypoglycemic condition. These results show the vulnerability of the cerebellum to ethanol, especially in the developmental period [94]. Studies have shown that ethanol inhibits cell survival mediated by neurotrophic factors, which affects granule cell migration and alters Purkinje cell function [Reviewed in [95, 100].

BDNF, NT-4, and TrkB are highly expressed in the cerebellum. There is evidence that developmental outcomes of ethanol exposure are mediated by alteration

of neurotrophins [96] in an area- and time-specific manner [Reviewed in [97]]. Allelic variation of BDNF might play a role in greater total and gray matter volume of the cerebellum in alcohol-dependent families [98]. Prenatal ethanol exposure caused a decrease in BDNF expression in the embryonic rat brain [99]. Ethanol reduces BDNF and NT-3 secretion in the neonatal rat granule cells. Reduced neurotrophin levels increase after treatment by vitamin E [100]. Ethanol influences neurotrophin receptor expression, including TrkA, TrkB, TrkC, and p75NTR. After ethanol treatment in the early postnatal rat cerebellum, reduction of these receptors was reported [101].

Ethanol prevents BDNF activity in the cerebellum, and this leads to damage to cerebellar Purkinje cells, which may occur via impairment in regulation of BDNF, TrkB receptor, or related signaling pathways [102]. BDNF is also necessary in the development and migration of cerebellar granule cells in the postmitotic period [103]. In vitro experiments on granule cells showed that ethanol inhibits the BDNF-stimulated phosphorylation of extracellular signal-regulated protein kinase (pERK) activation in neurons [104]. BDNF stimulates AP-1 in cerebellar granule neuron culture, and PI3K/Akt and JNK pathways interfere with this effect. Ethanol suppresses the PI3K/Akt and JNK pathways and also AP-1 activity linked to BDNF [105]. This change in BDNF signal transduction reflects developmental abnormalities that result from ethanol consumption [104]. Reduction in BDNF is associated with sensitivity to neural cell degeneration [106]. It has been shown that the levels of BDNF, TrkB receptor mRNA expression [107], TrkC receptor [108], and NGF in the cerebellum decrease on postnatal day 4 or 5 [109].

Similar to other neurotrophic factors, NGF plays a critical role in the development of different parts of the brain such as the cerebellum. Studies have shown that ethanol exposure in neonatal rats reduces existing NGF receptor levels in Purkinje cells [110]. Purkinje cells showed the most deleterious effects of ethanol during early neonatal development of the cerebellum [108]. This effect influences neurotrophin signaling [109] and upregulates proapoptotic molecules [96, 111], which cause Purkinje cell loss via apoptosis [112]. Cerebellar granule cell development is impaired after prenatal ethanol exposure. Src family kinases (SFKs) are signaling molecules that trigger axon growth. Ethanol inhibits SFK and disrupts granule neuron outgrowth. However, the effect of ethanol on BDNF-dependent axon growth and the ERK1/2 pathway in granule cells is controversial [113]. Studies in mice with fetal alcohol syndrome suggested that mice that overexpress NGF have less Purkinje cell degeneration. The anterior lobe vermis is widely affected, and lobules IX and X are also affected by ethanol in both human and rodent fetal alcohol syndrome. Higher levels of TrkA receptors and p75NTR deliver more stability against degeneration in the posterior part of the cerebellum [114]. Long-term but transient exposure to ethanol (6 and 9 months) is accompanied by different NGF levels in the brain. No change in NGF levels of the cerebellum was reported in this survey [115]. Induced damage by ethanol toxicity in the developing cerebellum was attenuated by estradiol. It has been shown that estradiol protects Purkinje cells exposed to ethanol and increases BDNF mRNA after ethanol exposure [116].

Another affected neurotrophic factor in developmental ethanol neurotoxicity is GDNF. An explant culture model of this neurotoxicity in the rat cerebellum showed that ethanol exposure caused decreased GDNF release but did not change its mRNA expression [117]. These researchers also observed a preventive effect of exogenous GDNF against apoptotic cell death signaling that was caused by ethanol treatment in a cellular model [118]. Chen et al. then showed that GDNF along with netrin-1 and L1, an adhesion molecule in neural cells, has converging effects on activation of the SFK-cas-ERK1/2 pathway to promote axonal outgrowth. Ethanol disrupts this pathway and inhibits axonal arborization in cerebellar granule cells [113].

Medulloblastoma

Medulloblastoma is the most common pediatric brain tumor in the cerebellum of infants and children [43, 119] (see chapters "Primary Pediatric Brain Tumors of the Posterior Fossa and Primary Pediatric Brain Tumors of the Posterior Fossa"). Marchetti et al. suggested a critical role for NTs and their receptors in the invasive feature of human medulloblastoma [120]. Although as previously mentioned, Trks are important factors for neuronal survival, but NGF/TrkA signal transduction is accompanied by suppression of medulloblastoma cell proliferation [121] and induction of cell death [122–124]. Interaction of the cytoplasmic adaptor protein CCM2 with the TrkA receptor is necessary in this pathway. The mediator of TrkA-CCM2 death signaling in medulloblastoma cells is STK25, which is a germinal center kinase class III (GCKIII) kinase (STK24, STK25). Reduction of STK25 prevents medulloblastoma cell death induced by NGF-TrkA [125]. Another study involving a cellular model of medulloblastoma reported induction of cell death after activation of TrkA by NGF through macropinocytosis [126]. Valderrama et al.'s findings confirmed that induction of TrkA expression resulted in either medulloblastoma cell differentiation or apoptosis [127]. Whole genome microarray analysis revealed that TGFβ is a potent factor that influences progression and metastasis of tumor cells in [42]. Gate et al. showed that obstruction of TGFβ signaling almost completely eliminates T regulatory cells and improves CD8(+)/killer cell function to eradicate tumor cells [128].

Schizophrenia

Schizophrenia, which is classified as a late-onset neurodevelopmental disorder [78], is a hereditary (80%) chronic mental disease [129] with cognitive abnormalities [130]. Involvement of cortico-cerebellar connections in cognition has been suggested by brain imaging studies [131]. Researchers have suggested that schizophrenia may be related to cerebellar anomalies [132] including a size and density decrease

of Purkinje cells and remodeling of synaptic protein expression in the cerebellum [133]. There is growing evidence of a role for neurotrophin in the pathophysiology of schizophrenia [134, 135]. Some studies showed the difference in plasma BDNF and NGF levels between schizophrenic patients and normal people. The levels of NGF in schizophrenia have been reported to be lower than in normal people [136–138], while there were no differences in BDNF or NGF levels in peripheral blood mononuclear cells (PBMCs) in patients and controls in the Martinez study [139]. However, Paz et al. reported increased BDNF levels in the cerebellar cortex of schizophernic patients [140]. In newly diagnosed psychosis patients, serum NGF levels decrease, and this may be a good biomarker in the diagnosis or screening for patients with schizophrenia [141–144]. A synaptic plasticity defect observed in schizophrenia may be associated with NGF and its receptor (NGFR). A positive association between schizophrenia and both the NGF rs6330 and the NGFR rs11466155 and rs2072446 SNPs was reported [144]. Alterations of neurotrophins in an animal model of schizophrenia have been confirmed. In animals injected subchronically with ketamine (Ket), which is a good model to study schizophrenia, Becker et al. reported that NGF, NT-3, and BDNF mRNA levels and their tyrosine kinase receptors changed in several brain regions and in the cerebellum [145]. A decrease in NGF levels in drug abusers was also reported. The role of neurotrophin in schizophrenia suggests that reduced levels of neurotrophins may increase the risk of psychosis in drug users [146].

Williams Syndrome

Williams syndrome (WS) is a rare neurodevelopmental disorder that affects 2–5/100,000 people [147], and it is caused by a 1.6 Mb deletion on chromosome 7 (7q11.23) [72]. This syndrome is characterized by an enlarged cerebellum and mild-to-moderate mental retardation with a deficit in visuospatial processing and an oversensitivity to sound [72]. NGF levels in the serum of WS patients are higher than in normal people, and they remain continuously higher during childhood. This is on contrast to normal people, who have a higher serum NGF only in early childhood [148].

Other Cerebellar Neurodevelopmental Disorders

There are some other disorders of the cerebellum such as Joubert syndrome [6], Dandy–Walker malformation [149], pontocerebellar hypoplasia [149], cerebellar vermis hypoplasia [149], and developmental dyslexia [5, 150], that occur during development, but to our knowledge, there is no data available about any association of neurotrophic factors with these conditions, which suggests new areas of research.

The role of neurotrophic factors on the cerebellar neurodevelopmental disorders is summarized in Table 1.

Table 1 The role of neurotrophic factors on the cerebellar neurodevelopmental disorders

CND	Study model	NF/R	Effect	Reference
ADHD	Human case control	BDNF	Plasma protein level increased in child patients	[55, 56]
		BDNF	Serum protein level unchanged in child patients	[57]
		BDNF	Serum protein decreased in adult Caucasians	[58]
		NGF, BDNF	No significant changes in Turkish population	[26]
		GDNF, NT-3	Serum protein level was higher in Turkish population	
		NGF	Serum protein level increased in child patients	[61]
ASDs	Rat infected Borna disease virus	NGF, BDNF, and NT-3 and their respective Trk receptors	Unchanged in the cerebellum	[68]
	Human case control	NT-3, NT4/5	Decreased in the spot-blood of newborns	[69]
	Postmortem human case control	NT-3	Increased in cerebellar samples	[10]
	Mouse model of Rett syndrome	NGF	Decreased or unchanged on the cerebellum	[31]
	Human case-control Rett syndrome	NGF	Decreased with age	[84]
Congenital ataxia	Mouse model	BDNF	Decreased mRNA level in granule cell layer	[90]
		NGF	Unchanged	
Ethanol neurotoxicity	Rat cerebellar vermis	TrkA, TrkB, TrkC	Decreased	[111]
	Neonatal rat cerebellar granule cells	BDNF, NT-3	Decreased secretion	[100]
	Neonatal rat cerebellum	BDNF, NGF, TrkA, TrkB, TrkC, and p75NTR	Decreased expression	[101, 107–110]

(continued)

Table 1 (continued)

CND	Study model	NF/R	Effect	Reference
	Granule cells	BDNF	Inhibit its activation effect on ERK pathway	[104]
	Cerebellum of Short-term ethanol exposed mouse	NGF, TrkA	Increased mRNA and protein level	[151]
		BDNF		
		TrkB, p75NTR	Unchanged	
	Explant culture of rat cerebellum	GDNF	Decreased release despite unchanged mRNA expression	[117]
	Cerebellar granule cells	GDNF	Ethanol inhibited its activation effect on SFK-Cas-ERK1/2 pathway to promote axonal outgrowth	[113]
Medulloblastoma	MB cells	NGF, TrkA	Suppressed their proliferation	[121]
		NGF, TrkA	Induced apoptosis	[124, 126]
	MB patients	TrkA		[123]
	Whole genome microarray on MB tumors	TGF-ß	Influence progression and metastases	[42]
	MB transgenic mouse	TGF-ß	Obstruction of TGF-ß leads to restriction of MB	[128]
Schizophrenia	Human case control	NGF	Plasma protein level decreased	[138, 143]
		NGF, BDNF, TrkA,	Unchanged in PBMCs	[139]
		TrkB	Differential expression of its different isoforms in PBMCs	[139]
		BDNF	Unchanged in the cerebellar cortex	[140]

ADHD attention deficit hyperactivity disorder, *ASDs* autism spectrum disorders, *BDNF* brain-derived neurotrophic factor, *Cas* Crk-associated substrate, *CND* cerebellar neurodevelopmental disorder, *ERK* extracellular receptor kinases, *GDNF* glial-derived neurotrophic factor, *MB* medulloblastoma, *NGF* nerve growth factor, *NT-3* neurotrophin 3, *NT-4/5* neurotrophin 4/5, *NF/R* neurotrophic factor/receptor, *PBMCs* peripheral blood mononuclear cells, *p75NTR* P75 neurotrophin receptor, *SFK* Src family kinases, *TGF-β* tumor growth factor-β, *Trk* tropomyosin receptor kinase

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Apoptosis, Autophagy, and Unfolded Protein Response and Cerebellar Development

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Abstract Development is an evolutionary process that is tightly regulated in mammalian species. Several different cascades are involved in different stages of development. Among these mechanisms, apoptosis, autophagy, and unfolded protein response play critical roles in regulation of development by affecting the cell fate. All of these pathways are involved in regulation of cell number via determining the life and death cycles of the cells. In this chapter, we first explain the brief mechanisms that are involved in regulation of apoptosis, autophagy, and unfolded protein response, and later, we briefly describe how these mechanisms play roles in general development. We then discuss the importance of these pathways in regulation of cerebellar development.

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Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
Apaf-1	Apoptotic protease activating factor 1
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATGs	Autophagy-related proteins
Bcl-2	B-cell lymphoma protein 2
BiP	Immunoglobulin heavy chain binding protein
bZIP	Basic leucine zipper protein
CAD	Caspase-activated DNase
CARD	Caspase recruitment domains
Caspases	Cysteinyl aspartate proteases
CERKL	Ceramide kinase-like
CGS	Cerebellar granule cells
CHOP	C/EBP homologous protein
CMA	Chaperone-mediated autophagy
Cyt *c*	Cytochrome *c*
DED	Death effector domain
DIABLO	Direct IAP binding protein with low pI
DISC	Death-inducing signaling complex
DTT	Dithiothreitol
EGL	External granule layer
eif2α	Eukaryotic initiation factor 2 alpha
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
ERSE	ER stress response element
FADD	Fas-associated death domain
GCPs	Granule cell precursors
GL	Granule layer
GNPs	Granule neuron precursors
GRPs	Glucose-regulated proteins
HA	Hemagglutinin
HD	Huntington's disease
HSPs	Heat shock proteins
HSR	Heat shock response
HtrA2	High temperature requirement protein A
IGL	Internal granule layer

IRE1	Inositol-requiring transmembrane kinase/endoribonuclease 1
LC3	Microtubule-associated protein light chain 3
MPT	Mitochondrial permeability transition
mTOR	Mammalian target of rapamycin
NOND	Naturally occurring neuronal death
pcd	Purkinje cell degeneration
PCD	Programmed cell death
PD	Parkinson's disease
PDI	Protein disulfide isomerase
PE	Phosphatidylethanolamine
PERK	Double-stranded RNA (PKR)-activated protein kinase-like eukaryotic initiation factor 2α kinase
PI3K	Phosphatidylinositol 3-kinase
PMDs	Protein misfolding disorders
PMT	Permeability membrane transition
PrDs	Prion-related diseases
ROS	Reactive oxygen species
Smac	Second mitochondria-derived activator of caspase
TGF	Transforming growth factors
TRADD	TNF receptor-associated death domain
ULK	Unc-51-like kinase
VZ	Ventricular zone
XBP1	X-box binding protein-1
XBP1s	Spliced XBP1
XBP1U	Unspliced XBP1

Introduction

One of the most important issues in the developmental process during the early mammalian embryonic period is understanding how the undistinguishable cells in the early embryo later develop to different fates and how different mechanisms that are involved in cell fate regulate this process. Besides existing models, many recently revealed molecular, cellular, and developmental factors have significant functions in determining cell position, cell polarity, and transcriptional networks in cell fate parameters throughout preimplantation. It is well known that the structuring process known as compaction provides the initiating signal for cells to start differentiation and arranges the initiation of developmental cascade. Here, we provide an overview of the three mechanisms that are involved in determining cell fate including apoptosis, autophagy, and unfolded protein response (UPR), and later we explain how these mechanisms are involved in regulation of cerebellar development. These mechanisms are the major determining steps that are involved in proper

cell fate specification in the early mammalian embryo, and they play essential roles in development.

Introduction to Programmed Cell Death (I) (Apoptosis)

The term apoptosis was first introduced by Kerr, Wylie, and Currie in 1972 to define a distinct mode of cell death under physiological conditions in hepatocytes [57, 76]. During the early process of this type of cell suicide, cellular content is condensed and cell shrinkage is observed. Apoptosis is a genetically conserved pathway in all metazoans such as in nematodes, insects, and mammals [25, 51, 96]. Typical morphological features of apoptosis include chromatin condensation (pyknosis), inter-nucleosomal DNA fragmentation, membrane blebbing and budding, and finally formation of small membrane-bound vesicles, called apoptotic bodies [28, 76] (Fig. 1). In contrast to necrotic death, membrane integrity is retained during apoptosis, and phosphatidylserine, a plasma membrane phospholipid, localizes from the inner side to the outer layer, acting as an "eat me" signal; the cell is then rapidly detected and engulfed by macrophages [25, 28, 51] (Fig. 1).

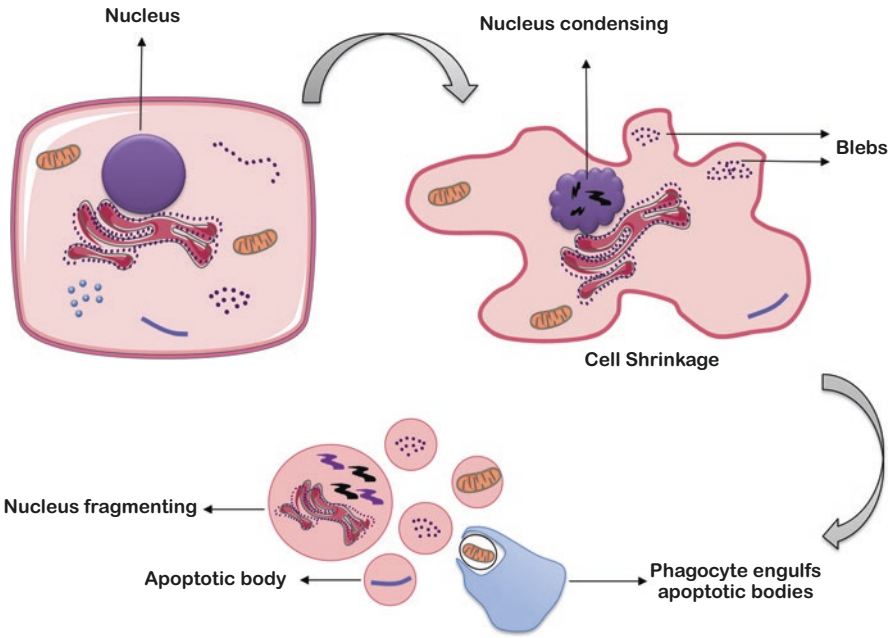

Fig. 1 Cellular morphology changes during apoptosis. Apoptosis is an ATP-dependent mechanism which includes chromatin condensation (pyknosis), inter-nucleosomal DNA fragmentation, membrane blebbing and budding, and finally formation of small membrane-bound vesicles, called apoptotic bodies

Caspases Are Central Initiators and Executioners of Apoptosis

Our understanding of the molecular components of apoptosis emanated from genetic studies of programmed cell death (PCD) in the nematode *Caenorhabditis elegans*. Two genes, *ced-3* and *ced-4* (cell death abnormal), are essential to determine which cells undergo PCD during *C. elegans* development. The protein encoded by the *C. elegans ced-3* gene is similar to the amino acid sequence of mammalian interleukin-1β (IL-1β)-converting enzyme (ICE), a member of the family of *c*ysteinyl *asp*artate prote*ases* (caspases) [24]. In the living cells, caspases exist as inactive zymogenes (procaspases) that contain the N-terminal pro-domain followed by a large and a small subunit. Upon activation of procaspases, the pro-domain is frequently removed, and proteolytic processing occurs between the other domains so that the small and two large subunits are associated in a heterodimer (Fig. 2) [34, 98]. To date, 14 different caspases have been identified in mammals, and their nomenclature is based on the order of their publication. For example, ICE is the first mammalian caspase and is named caspase-1. Pro-apoptotic caspases are divided into the initiator procaspase group (i.e., procaspase-2, procaspase-8, procaspase-9, and procaspase-10) and into the effector (executive) procaspase group (i.e., procaspase-3, procaspase-6, and procaspase-7) [34, 98]. Following activation, the executive caspases degrade most vital proteins in the cells, leading to disruption of the cytoskeleton, intracellular transport, and nuclear envelope and signal transduction that ultimately cause the morphological and biochemical changes of apoptosis. For example, the nuclear scaffold proteins (lamins), the cytoskeleton protein (alpha-fodrin), the plasma membrane blebbing mediator (gelsolin, act as a nucleus for

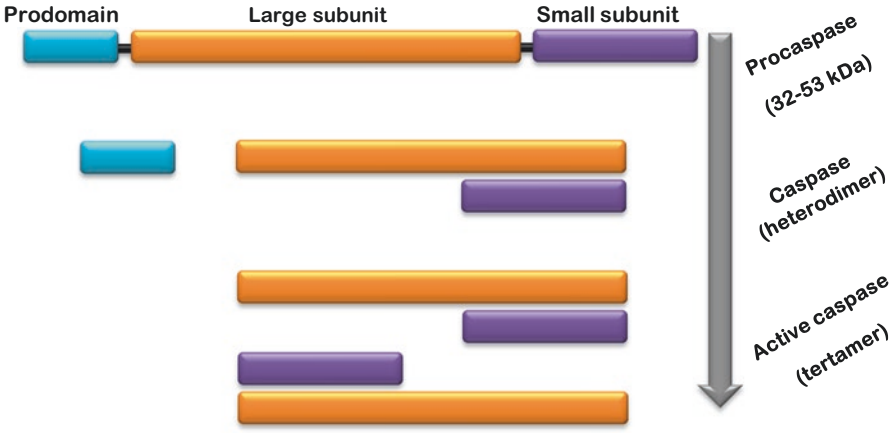

Fig. 2 Structure and activation of caspases. Inactive form of caspases (procaspases) includes three subunits. Mechanism of caspase activation is initiated by autoproteolysis of Asp residues into large subunits followed by assembly of active heterotetramers. Following proteolysis cleavage, procaspase can be in close proximity to each other and therefore is assumed to activate each other

actin-depolymerizing enzyme), and poly(ADP-ribose) polymerase (PARP) are targeted proteins that are cleaved during apoptosis [17]. In addition, caspase-activated DNase (CAD) that mediates DNA ladder hallmarks of apoptosis is activated by caspase-3 and caspase-7 cleaving the CAD inhibitor. Whereas activation of initiator caspases is mediated through binding of their pro-domains to adaptor molecules via death effector domains (DED) or caspase recruitment domains (CARD), activation of executive caspases occurs through proteolysis at internal Asp residues into large subunit followed by assembly of active heterotetramers [25, 98] (Fig. 2).

Molecular Pathways of Apoptosis

The apoptosis cascade is initiated by three major signaling pathways, including the cell death receptor pathway, mitochondrial pathway, and endoplasmic reticulum (ER) stress-induced pathway. In all pathways, caspase-3 is the main executive caspase that is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10) [25, 28, 34, 51, 76, 98].

Cell Death Receptor Pathway

The extrinsic pathway is mediated by activation of death receptors, which are transmembrane receptors that transmit apoptotic signals from the cell surface to the intracellular signaling pathways via receptor–ligands interactions [71, 98]. Death receptors involve Fas (CD95) and TNF receptor (TNFR1) as well as TRAIL receptors DR4 and DR5 (TNF-related apoptosis-inducing ligand receptor 1 and 2, TRAIL-R1 and TRAIL-R2). Their corresponding ligands are called TNF, Fas ligand (FasL), and Apo2L (TRAIL), respectively. The sequences of events that define the death receptor pathway of apoptosis are best characterized using the FasL/FasR and TNF/TNFR models [17, 25, 51] (Fig. 3). In this scenario, the first step is trimerization and clustering of receptors by related ligand. Upon ligand binding, cytoplasmic adapter proteins are recruited via intracellular receptor death (DD) domain, such as Fas-associated death domain (FADD) and TNF receptor-associated death domain (TRADD). At this point, FADD or TRADD is associated with procaspase-8 via dimerization of the death effector (DED) domain, thereby forming the death-inducing signaling complex (DISC) [25, 28, 51, 98]. The increase in the local concentration of procaspase-8 at the DISC resulted in their autocatalytic activation (Fig. 3). Activated caspase-8 finally cleaves and actives the effector caspase-3 that leads to initiating the execution phase of apoptosis (Fig. 3).

The cells that need DISC-mediated signals to complete the cascade are classified as type I cells, while the cells that require the contribution of a mitochondrial pathway to complete the apoptotic process are classified as type II cells [100]. In type II cells, the receptor-mediated signaling is not strong enough to activate caspase for execution of apoptosis so that the signal needs to be amplified via mitochondria-dependent apoptotic pathways [25, 34, 51, 98, 116]. The link between cell death receptor path-

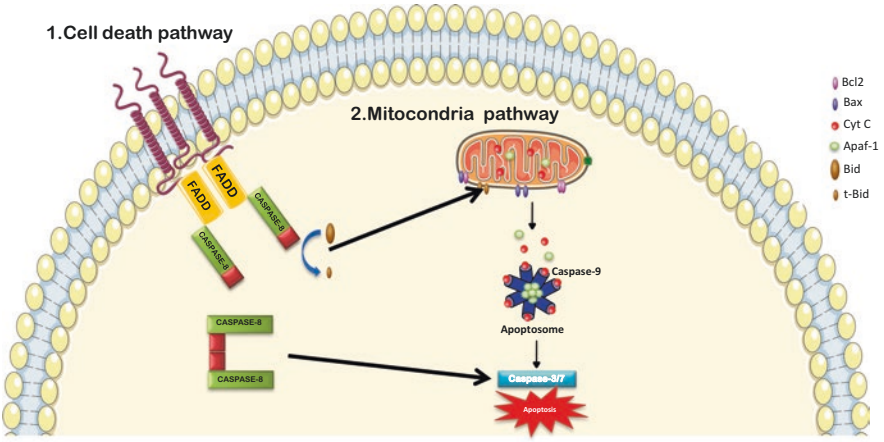

Fig. 3 Intrinsic and extrinsic apoptosis pathway. Extrinsic pathway (1) is commenced via death receptors which later activates initiator caspases (like caspase-8) with subsequent Bid protein truncation (link to mitochondria) or directly activates caspase-3 and caspase-7 (executor caspases) and induces nuclear fragmentation. Intrinsic pathway is initiated via mitochondria following activation of caspase-9 and caspase-3/caspase-7 activation

way and the mitochondria is provided by the Bcl-2 family member Bid. Bid is cleaved by caspase-8 and in its truncated form (tBID) translocates to the mitochondria where it acts together with the pro-apoptotic B-cell lymphoma protein 2 (Bcl-2) family members Bax and Bak to induce the release of cytochrome *c* (cyt *c*) and finally turn on the mitochondrial apoptosis pathway [25, 51, 76, 98].

Mitochondrial Pathway

The mitochondrial pathway, also called the intrinsic pathway, is initiated from inside the cell. Various stimuli such as growth factor withdrawal, DNA damage, hypoxia, and oxidative stress can induce apoptosis through this cascade [28, 34, 98, 116]. Cellular stresses cause an increase of permeability in the outer mitochondrial membrane and opening of the mitochondrial permeability transition (MPT) pore, which is controlled by members of the Bcl-2 family proteins [25, 51, 98]. Bcl-2 family proteins are defined by the presence of conserved Bcl-2 homology domains (BH1 to BH4). Up to 30 Bcl-2 family genes have been identified in mammals, which have either pro-apoptotic or anti-apoptotic functions. Some of the anti-apoptotic members including Bcl-2, Bcl-XL, Bcl-w, BAG, and Mcl-1 possess all domains, from BH1 to BH4 [72, 81]. The pro-apoptotic family proteins can be divided into two subgroups: the group that includes proteins with BH1 to BH3 domains (e.g., Bak, Bax, and Bok) and the group that involves proteins with BH3 domain (e.g., Bad, Bid, Bik, BNIP3, Bim, Bmf, Blk, Hrk, Noxa, Puma, and Spike) [95, 116]. BH3-only proteins are thought to interfere with the fine-tuned balance of homo- or hetero-oligomerization between pro-apoptotic multi-domains (e.g., Bax/Bak)

and anti-apoptotic members (e.g., Bcl-2/Bcl-XL) [72, 81] (Fig. 3). In general, oligomers of Bak, Bax, and Bok can form channels by themselves to induce permeability membrane transition (PMT) [4]. Bad can also heterodimerize with some members of anti-apoptotic Bcl-2 family proteins and thereby neutralize their inhibitory effects on mitochondrial pro-apoptotic Bcl-2 members [4, 72]. Puma and Noxa are also involved in *p53*-mediated apoptosis [18, 98]. Bcl-2 family proteins control the release of the mitochondrial proteins cytochrome c (cyt c), second mitochondria-derived activator of caspase (Smac)/direct IAP binding protein with low pI (DIABLO), and Omi/high temperature requirement protein A (HtrA2) into the cytoplasm [4, 6, 98]. Cytoplasmic cyt c binds to monomeric apoptotic protease activating factor 1 (Apaf-1), which then, in the presence of dATP, initiates oligomerization to form a complex wheel-like structure with sevenfold symmetry called an apoptosome [2, 6, 25, 98] (Fig. 3). This type of procaspase-9 clustering leads to caspase-9 activation, which subsequently actives downstream executive caspases such as caspase-3, caspase-7, and caspase-6 and ultimately leads to apoptosis. Smac/DIABLO and the serine protease HtrA2/Omi promote apoptosis by inhibiting inhibitors of apoptosis protein (IAP) activity [6, 77]. This family of anti-apoptotic proteins includes NAIP, c-IAP1, c-IAP2, XIAP, and survivin, the prototype of which was originally described in baculovirus. They can bind directly to caspases and inhibit their activity and are negatively regulated by proteins from the mitochondrial intermembrane [25, 51, 98, 107] (Fig. 3). Almost all of the morphological and biochemical features of apoptosis are mediated through the activity of caspases [17].

Apoptosis in Development

Apoptosis literally means “falling off” (as leaves drop from trees) in Greek, and this analogy suggests that the cell death is necessary for the life cycle of organisms [30, 37, 58, 76]. An example of the impact of PCD on development is seen in lymphocytes. Most lymphocytes die via apoptosis as a result of negative selection or genetic rearrangement, thereby verifying the constant cellular pool of functional immune cells and lymphocyte numbers [97]. Moreover, apoptosis is critical for development of reproductive organs [85]. Apoptotic processes are widely involved in regulation of proliferation, differentiation, development, and tissue homeostasis [26].

Autophagy and Its Role During Development

Autophagy

Autophagy is a tightly regulated catabolic process used by eukaryotes for recycling and degrading of organelles, proteins, and other cytoplasmic components, in a lysosomal-dependent manner [52]. Autophagy occurs in three typical forms

including microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA) [13, 60, 83]. Microautophagy and CMA are directly mediated by the lysosome to immediately degrade small cytosolic portions or chaperone-associated molecules, respectively. Macroautophagy (hereafter called autophagy) is responsible for the turnover of long-lived macromolecules and damaged organelles that are sequestered into the autophagosome, a double-membrane-bound vesicle originating from a precursor structure called the phagophore [33, 60, 74, 87] (Fig. 4). Autophagosomes are then fused with lysosomes and this forms the autophagolysosome (Fig. 4). In the final stage, the cargo is degraded by hydrolases in the autolysosome, and the products are transported back to the cytosol by lysosomal permeases [121].

The molecular components of this pathway were first discovered in the yeast *Saccharomyces cerevisiae* and include autophagy-related proteins (ATGs) [91]. Most autophagy stimuli converge at the phosphatidylinositol 3-kinase (PI3K)–Akt–mammalian target of rapamycin (mTOR) pathway that is the best characterized modulator of autophagy in most cells [11, 60] (Fig. 5). This signaling pathway is known to play a vital role in multiple cellular functions such as proliferation, adhesion, migration, survival, and invasion [56, 117]. The PI3K–Akt–mTOR pathway integrates signals from growth factors, energy, and nutrients to adjust proliferation and cell growth through various cellular mechanisms [75, 117]. The serine–threonine protein kinase Akt, also called protein kinase B, is upstream of mTOR and the downstream effector of PI3K. Inactivation of mTOR complex1 by starvation

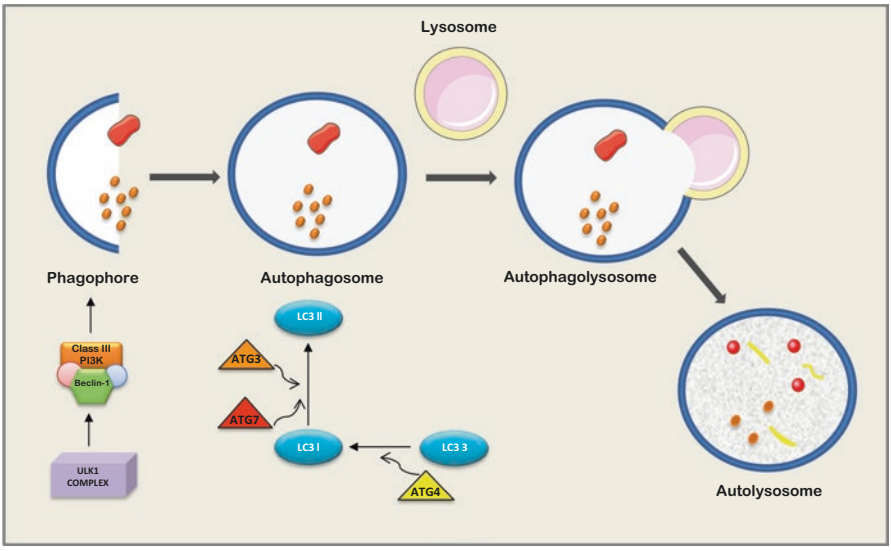

Fig. 4 Schematic representation of autophagy pathway. Autophagy is a process for the degradation and recycling of cellular compartments in lysosomes which includes phagophore, autophagosome, autophagosome, and autolysosome

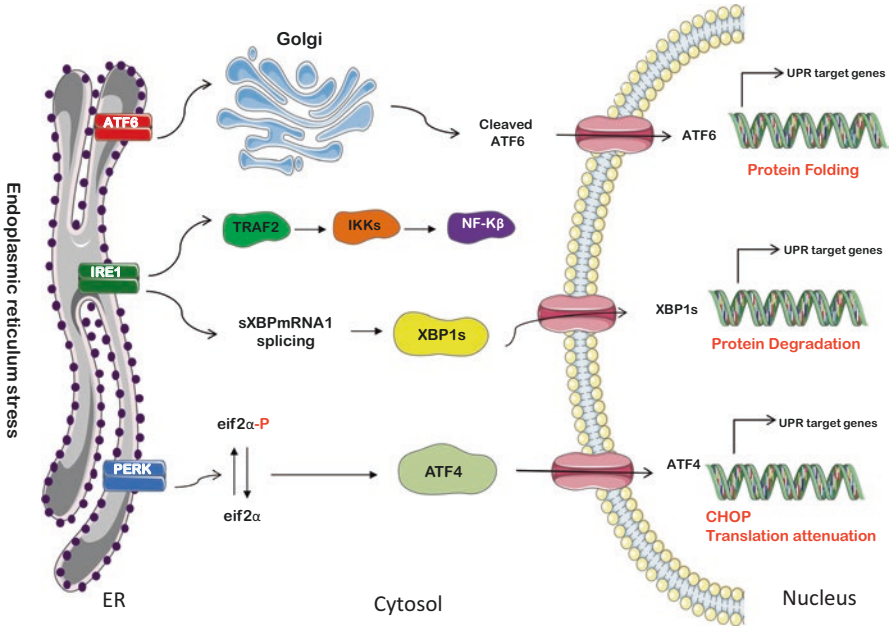

Fig. 5 Schematic representation of ER stress and unfolded protein response. ER stress activates UPR proteins (e.g., PERK, ATF6, and IRE1) in the endoplasmic reticulum. Briefly activated PERK promotes ATF4 activation via phosphorylation of eif2α. Activation of IRE1 arm of UPR induces XBP mRNA splicing in the cytoplasm, subsequently leading to activation of UPR target genes. ATF6 arm is initiated via cleavage of ATF6 in the Golgi which is later targeted to the nucleus and induces expression of UPR-responsive genes

conditions activates Unc-51-like kinase (ULK), which initiates the autophagy process; however, under normal nutrient conditions, mTOR complex1 phosphorylates ULK1/ULK2 and Atg13 to inhibit initiation of the autophagy pathway [99, 117]. Therefore, core machinery for the initiation stage during autophagy induction is the ULK1/ULK2 complex, which consists of ULK, Atg13, and FIP200. Upon initiation of autophagy, a complex nucleation arises when the PI3K complex binds to its core units, such as Beclin-1 (the human orthologue of murine Atg6) [73, 74]. This complex resides on the isolated membrane and facilitates recruitment of other ATGs to the unit (Fig. 5). During autophagosome elongation and maturation, two ubiquitin-like conjugation systems are involved: the microtubule-associated protein light chain 3 (LC3) system and the Atg12 system. LC3 is first cleaved by ATG4 to form LC31. Phosphatidylethanolamine (PE) is then conjugated to LC3I by Atg7 and Atg3, and it creates LC3-II that can stably insert into the autophagosomal membrane [33, 35, 63, 87].

Role of Autophagy in Development

There is emerging evidence that autophagy plays critical roles in differentiation and development [1]. The autophagy pathway can induce rapid cellular changes (e.g., protein and organelle turnover) that are necessary for proper differentiation and/or development. Most autophagy-defective organisms show severe problems in differentiation [12]. Additionally, autophagy is important for survival during neonatal starvation and cell differentiation during lymphopoiesis, erythropoiesis, osteogenesis, and adipogenesis [1, 12, 86]. The Atg7-deficient mice showed severe anemia because there is a lack of sufficient erythropoiesis. It has been suggested that erythroid differentiation depends on autophagy for mitochondria removal [89]. Adipocytes mainly harbor lipid droplets that have been identified as a substrate for autophagy [23]. In addition to the differentiating functions, autophagy is also crucial for survival and viability of terminally differentiated cells such as neurons. Autophagy knockout mice showed uneven numbers of neural stem/progenitor cells which resulted in a delay in development. The phenotype of Ambra1 and ULK1 mutant mice confirmed the importance of autophagy and plausible mechanisms during development of the nervous system [27]. Ambra1 is a vertebrate-specific protein that is highly expressed in the nervous system and positively regulates autophagy by promoting Beclin-1 binding to Vps34 [27]. ULK1 is an ATG protein involved in the initiation of autophagy, and its deficiency leads to defects in terminal neuronal differentiation and causes abnormal axonal formation in the cerebellar granule neuron [109]. Moreover, autophagy is crucial for vertebrate development at some time points during embryogenesis. In the embryonic period, the placenta provides energy for the mammalian embryo but after birth, transplacental nutrient supply is disconnected and embryos face starvation until the supply can be restored through milk nutrients. In this condition, autophagy is induced approximately 3 days ± 12 h after birth [65]. Confirming these reports, Atg5 null mice are normal at birth, but die within 1 day after birth, highlighting the importance of autophagy for embryo development [65].

Endoplasmic Reticulum Stress and Unfolded Protein Response

The presence of two glucose-regulated proteins (GRPs), GRP78 and GRP94, with molecular weights of 78 and 94 kDa, respectively, were discovered in the endoplasmic reticulum (ER) of mammalian cells in 1987. They can form stable associations with a variety of proteins retained in the ER because of underglycosylation or other conformational changes [67]. These proteins are induced by some stress conditions including glucose starvation, treatment with cellular glycosylation inhibitors, calcium ionophores, or amino acid analogues [67]. The major difference between these

proteins and heat shock proteins (HSPs) is that GRPs are not induced by increasing temperature [67]. GRP78 was shown to have some activity like immunoglobulin heavy chain binding protein (BiP) [90]. It can also permanently bind to a variety of malfolded/misfolded proteins that accumulate within the ER and/or transiently in nascent, wild-type secretory and transmembrane proteins. For the first time in 1988 while studying simian cells, it was reported that only malfolded proteins (e.g., influenza virus hemagglutinin (HA)) had their transport from the ER blocked, which can induce GRPs 78 and 94 synthesis regardless of their abnormal glycosylation state [64]. It has also been shown that the highly conserved *GRP* element, which is important for the basal level and induced *GRP78* expression, is a 10-bp region that contains a CCAAT motif in DNA. However, this element alone is not sufficient for promoter activity, but a 40-bp region (−129 to −90) that contains this motif is essential for mediating basal levels and stress inducibility of the *GRP78* promoter. It has also shown that the transcription factor CTF/NF-I is able to transactivate the *GRP78* promoter through interaction with this CCAAT motif [120].

Less than 25 years ago, an FK506/rapamycin-binding protein was found to be encoded by a mammalian *FKBP-13* gene, which localizes in the ER lumen. A homologue of mammalian *FKBP-13* in the ER lumen, the FKB2 gene, is encoded by *S. cerevisiae*. FKB2 mRNA levels increase in response to the accumulation of unfolded proteins in the ER, which can be caused by blocking the N-glycosylation and/or treating the cells with tunicamycin. However, blocking other steps in secretion has no effect on FKB2 mRNA levels. It was then shown that a 21-bp UPR element located in the 5′-noncoding region of *FKB2* is responsible for this increase in the *FKB2* mRNA level. The similarities in the regulation of *FKB2* and other ER chaperone genes (yeast *KAR2*, mammalian *GRP78* or BiP, and *GRP94*) suggest that FKBP-13 may play a role in protein trafficking in the ER [93]. In addition to tunicamycin, other inducers of GRPs include dithiothreitol (DTT) as a sulfhydryl reducing agent, amino acid analogues, severe glucose depletion, and oxygen, which increase the protein flux, decrease Ca^{2+} levels, and disrupt lipid homeostasis to induce the UPR in the cells [68, 112, 114].

Thus, UPR is a regulatory mechanism by which cells control levels of misfolded proteins in the ER. The UPR is currently characterized in all cell types, including normal neurons, with an emphasis on its importance in neurodegenerative diseases. It has been shown that UPR signaling modulates neurodegeneration depending on the disease context [43].

In metazoans, UPR consists of three parallel arms, which are characterized by their stress sensor proteins: (1) inositol-requiring transmembrane kinase/endoribonuclease 1 (IRE1), (2) activating transcription factor 6 (ATF6), and (3) double-stranded RNA (PKR)-activated protein kinase-like eukaryotic initiation factor 2α kinase (PERK). Each of these UPR sensors binds to the BiP as the ER luminal chaperone (Fig. 5) [104, 112].

The IRE1 pathway is considered to function as a major and the most conserved arm of the UPR from yeast to humans [104, 112]. IRE1α and IRE1β have been known as two homologues of mammalian IRE1. While IRE1α is expressed in all cells and tissues, and is a main mediator of UPR signaling, IRE1β is expressed only

in the intestinal epithelium [112]. Activated IRE1α, which shows the endoribonuclease activity, cleaves a 26-base fragment from the mRNA encoding the X-box binding protein-1 (XBP1) [70, 127]. The *Xbp1* mRNA before and after splicing is translated into unspliced XBP1 (XBP1U) and spliced XBP1 (XBP1S), respectively. XBP1s as a potent transcription factor targets a wide variety of genes encoding proteins involved in ER membrane biogenesis, ER protein folding, ER-associated protein degradation (ERAD), and protein secretion [127]. Unspliced XBP1 mRNA is constitutively translated into XBP1U [88].

The role of ATF6, as a basic leucine zipper (bZIP) protein that belongs to the type 2 transmembrane glycoprotein family, has been introduced as another arm of the mammalian UPR. It has an important role as a putative ER stress response element (ERSE)-binding protein, which was introduced in 1998 by Yoshida et al. [126]. They showed that ATF6 was constitutively expressed in HeLa cells as a 90-kDa protein, but it was phosphorylated (p90ATF6) and converted to a 50-kDa protein (p50ATF6) by posttranslational mechanism is response to stress [126]. ATF6 is regulated by intramembrane proteolysis; ER stress induces the proteolysis of membrane-bound p90ATF6 and releases the soluble part, p50ATF6, allowing it to enter the nucleus. In the nucleus, p50ATF6 contains a bZIP domain and activates transcription of ER chaperone genes such as *GRP78* through ERSE in collaboration with a general transcription factor [41, 125]. It has been demonstrated that the XBP1, as a target of ATF6, is a mammalian substrate of such an unconventional mRNA splicing system and showed that only the spliced form of XBP1 (XBP1s) can effectively activate the UPR [127].

ATF6α and ATF6β are two distant homologues of ATF6 but both are ubiquitously expressed in all tissues [108]. They are cleaved during the ER stress response (ERSR); the resulting N-terminal fragments (N-ATF6α and N-ATF6β) enter the nucleus and bind to specific regulatory elements of the DNA, which results in the activation of transcription of ERSR genes related to ATF6, such as GRP78 [108]. It has been suggested that the relative levels of ATF6α and ATF6β may contribute to regulating the strength and duration of ATF6-dependent ERSR gene induction and cell viability. In addition, ATF6α is strong, but labile transcription factor, while ATF6β is a weak and stable transcription factor. A gel shift assay showed that they are competing with each other in binding to the GRP78 ERSE [108].

Harding et al. first introduced PERK in the mouse ER in 1999 [39]. PERK belongs to a family of protein kinases that in response to different cellular stresses regulates translation by phosphorylation of the α subunit of eukaryotic initiation factor-2 (eif-2α). Sood et al. then separated the rat homologue of PERK, which is pancreatic eif-2α kinase (PEK), from rat pancreas [105]. Protein synthesis and folding of the newly synthesized proteins into the correct three-dimensional structure are coupled in cellular compartments of the exocytosis pathway by a process that modulates the response to a stress signal from the ER [39]. The phosphorylation of eif-2α on serine residue 51 by PERK leads to the activation of the process to reduce rates of protein translation initiation during ER stress.

In some stress conditions such as amino acid starvation, protein synthesis is negatively regulated because of eif2α phosphorylation and its activation. In this signaling pathway, the mammalian eif2 kinases PERK and GCN2 repress translation of most

mRNAs but selectively increase translation of activating transcription factor 4 (ATF4), resulting in the induction of the downstream gene C/EBP homologous protein (CHOP) [38, 40, 79]. ATF4 is also activated by ER stress and other stimuli such as viral infection. However, there is no interaction between XBP1U and ATF4, which allows the cell to avoid undesired ATF4 degradation that is induced by XBP1U in response to non-ER stress [88]. Activation of ATF4 and CHOP negatively regulates mTOR via Redd1 expression in response to oxidative and ER stress [54].

UPR and General Development

Eukaryotic protein homeostasis, which is called proteostasis, refers to controlling all aspects of cells including health, organismal development and aging, as well as their protection against diseases, which is often influences protein synthesis (transcription/translation), degradation, conformation (folding/misfolding), protein interactions (quaternary structure, aggregation/disaggregation, and other protein–protein interactions), and trafficking (location of individual proteins). Thus, proteostasis affects specific cellular functions and enables differentiated cells to change their physiology in a surrounding media. Deficiency in the proteostasis results in some diseases like neurodegenerative, metabolic, and cardiovascular disorders and cancer. Some of these disorders are already developed at birth, but most occur upon aging [9].

As mentioned above, both development and aging are influenced by proteostasis. All protein processes such as protein folding, aggregation, degradation, and modification are the processes that affect protein function. Quality control systems in the cell control the balance between the abovementioned processes to achieve a high-quality protein suitable for growth and survival of the cell [7, 49, 101]. In addition to the metabolic enzymes, molecular chaperones, chemical chaperones, and some other small molecules affect the proteostasis. Several important processes including heat shock response (HSR) [44, 55, 113, 115] and UPR [9, 49] also regulate and control the proteostasis. For example, it has been shown that in cerebral pathological events such as ischemia, epilepsy, and trauma, some specific genes and proteins are activated, while some others may be inhibited in neuronal cells. Synthesis of a set of proteins, termed stress or HSPs, increases during ischemia as well as with heat shock treatment (hyperthermia), while the synthesis of most other proteins decreases [44]. Based on the time and region, there is also a significant difference between the kinetics of various HSPs [44, 45]. Northern blot analysis has indicated that there is differential induction of various classes of HSP mRNAs by ischemia. Within 4 h after ischemia, the HSP70 family mRNAs were induced and then rapidly decreased, while HSP27 and HSP47 mRNAs were maximally increased up to 24 and 48 h after ischemia, respectively. In addition, in situ hybridization showed that mRNAs of inducible HSP70s were localized in the core region of the infarct 2 h after ischemia, and at a relatively late period (4–8 h), they move to the penumbra region [45]. Because cerebral blood flow has been severely decreased in the ischemic center and the collateral circulation continuously provides some blood flow,

ischemic cell damage may progress from the ischemic center to the peripheral regions [44].

A growing body of evidence indicated the key role of UPR in normal neuronal function, and its distortion leads to neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and prion-related diseases (PrDs) [43]. Although clinical manifestations of these diseases are different, all involve the accumulation of misfolded proteins and are now classified as protein misfolding disorders (PMDs) [43, 106].

In addition to the ischemia and nervous system [36, 43, 46, 111], the role of UPR and proteostasis in general development [111] and the development of B- and T-cells [10, 32] and lens proteins [29, 123] have also been demonstrated.

The role of UPR activation during normal lens development and differentiation in the mouse has been studied. The lens of the eye, which is composed of epithelial and fiber cells, is a transparent structure that is responsible for focusing light onto the retina. Epithelial cells are found on the anterior surface, and after differentiation, fiber cells are formed at the lens equator. It has been shown that the expression of BiP and protein disulfide isomerase (PDI) was greatly increased in the newly forming fiber cells from embryonic lenses. These fiber cells also expressed the UPR-associated molecules XBP1, ATF6, p-PERK, and ATF4 during embryogenesis. In addition, XBP1s, cleaved ATF6, and p-eif2α have been detected in embryonic mouse lenses, suggesting that UPR pathways are active in this tissue [29]. In the lens epithelium of patients with cataract (high myopia-related or age-related cataract), the mRNA and soluble protein expression in both αA- and αB-crystallin were decreased. In addition, the protein levels of ATF6, p-eif2α, and p-IRE1α and the gene expression levels of spliced XBP1, GRP78, ATF6, and ATF4 were greatly increased relative to the normal control. These results suggest the significant loss of soluble α-crystallin and the activation of the UPR in the lens epithelium of patients with high myopia-related cataract, which may be associated with this type of cataractogenesis [123]. In the developing eyes, expression of ceramide kinase-like (CERKL) at both mRNA and protein levels was minimal, but it reached a peak at retinal maturity at 2 months of age in the mouse. The retina showed the highest level of CERKL expression, which reached its maximum in the adult retina [80].

Apoptosis and Cerebellum Development

In the cerebellum, the ventricular zone (VZ) and rhombic lip (RL) are the source of all cerebellar cell types. The VZ and the external granule layer (EGL) are able to proliferate, while the internal granule layer (IGL) and sub-ventricular zone are specified for migration. Purkinje cells and granule cells migrate from the VZ and EGL, respectively. Adult neurons are assumed to escape from apoptosis during proliferation or during early pre-mitotic migration. Thus, analysis of apoptosis in all parts of the cerebellum may help to identify different cell functions in development [3, 15].

Apoptosis of Stellate and Basket Cells

Progenitor cells such as stellate and basket cells are produced from the ventricular zone (VZ) postnatally. They reside in white matter in the first postnatal week, and in the days immediately following the first postnatal week, they proliferate more and migrate to the molecular layer. Their movement is completed in the third postnatal week. Apoptosis occurs during progenitor cell proliferation/migration as shown in the GAD67/GFP mice [78].

Apoptosis of Purkinje Cells

Purkinje cells migrate from the VZ and are placed between the EGL and IGL cells. They form a single-cellular layer [15] and are key cells in the cerebellum that are targeted in many neurological mutations in mouse models to study Purkinje cell death. Two periods of apoptosis occur in these cells: the first is the embryonic period and the second is the postnatal term. Regulation of Purkinje cell apoptosis occurs through the connection to climbing fibers. First, one climbing fiber interacts with several Purkinje cells during the first postnatal week in rats. Then, the climbing fibers fix their final connection. The Purkinje cells, which cannot react to climbing fiber, undergo apoptosis and are deleted. However, the apoptotic Purkinje cells can interact with climbing fibers and this causes them to retract. Thus, the connection between Purkinje cells and climbing fibers occurs on a one-to-one basis [15]. There are also many genes that interfere with regulation of Purkinje cell death. Two mouse models, Toppler and Woozy, are mutants in which PCD is observed in Purkinje cells by apoptotic pathways and sometimes with activation of autophagic mechanisms. This cell death may be different from formal PCD. Many of Purkinje cells will die during normal aging, using mechanisms similar to apoptosis [78].

Apoptosis of Granule Cells

Granule cells migrate from the EGL to the IGL part of the cerebellum. The apoptotic cells in IGL have been verified, and it was shown that they are postmitotic neuron cells that could not produce a right synaptic connection with Purkinje cells in the molecular layer [15]. During the first week of postnatal life, there is an established cell loss within the granule cell layer. A high number of granule neurons in both the mitotic and postmitotic regions of the EGL undergo DNA fragmentation [118]. The granule cell precursors (GCPs) are generated from the rhombic lip which is the source of external germinal zone (EGZ or EGL). GCPs, giving rise to GCs, first extensively proliferate and some of them start differentiating into mature GCs [20]. The first in vitro apoptosis model for the central nervous system was recently

identified, and it was shown that cerebellar granule cells undergo apoptosis when they are deprived of depolarizing levels of extracellular potassium [19]. Additionally, the in vivo correlation between apoptosis in cerebellar granule cells has been recently reported by Wood et al. [118], which shows DNA fragmentation in the granular layer of the cerebellum of the newborn rat. Cerebellar granule neurons are a perfect model system to study neuronal apoptosis because these neurons live and survive for weeks when they are maintained in depolarizing concentrations of potassium. However, they undergo apoptosis when cultured in low physiological potassium conditions. It has also been demonstrated that apoptosis of differentiated cerebellar granule neurons induced by potassium deprivation might be a neuronal death model after differentiation. They showed that during cerebellar development, target-related cell death in granule cells occurs [31, 102]. To comply with the existing hypothesis that during development transforming growth factors (TGF-β) might play a role in regulation of apoptosis in cerebellar neurons, these cytokines must be produced in a time- and location-dependent manner. It has been reported that TGF-β1, TGF-β2, and TGF-β3 accelerate neuronal apoptosis when maintained in a low physiological potassium medium, as assessed using quantitative DNA fragmentation, viability, and nuclear morphology. These data demonstrate that TGF-β might limit the expansion of neuronal precursor populations through boosting their apoptosis [20].

Additionally, there is evidence of a p53-independent apoptotic pathway for loss of cerebellar granule cells during development. This was demonstrated by the fact that neuronal precursors of apoptosis in the cerebellum of transgenic mice that lack functional p53 are similar to that in wild-type mice [119]. It has been previously suggested that elimination of postmigratory granule neurons during cerebellar development could be prevented by blocking their programmed death, further confirming the remarkable role of apoptosis in cerebellar development [122].

Apoptotic cells are identified as immature GCs and/or their GCPs. Analysis of apoptotic pathways has indicated that caspase-3 and caspase-9 are expressed in cerebellar germinal zones, and activation of caspase-3 is important for progenitor cell death, which is inhibited by a pan-caspase inhibitor. Thus, neural progenitors can activate a caspase-dependent apoptotic pathway. However, another experiment showed that caspase inhibitors could not prevent GCs from death. The experiments showed that caspase-3 is not activated during apoptosis of GCPs/pre-migratory GCs. Therefore, early neuronal death of GCPs/pre-migratory GCs may be caspase-3-independent. The naturally occurring neuronal death (NOND) of GCPs/pre-migratory GCs is possibly related to establishment of the correct ratio between GCs and Purkinje cells. The vast cell death in EGL neurons is related to folia formation during fissuration of the cerebellar cortex. The process of apoptosis does not occur synchronously in the cerebellum, and thus the number of apoptotic cells in the lobes is different. The second wave of apoptosis in GCs occurs in postmitotic neurons. The evidence has shown the specific cleavage of several caspases and PARP-1, the most biologically relevant substrate of caspase-3, occurs. The caspase/PARP-1 cleavage selectively occurs within the internal granule layer (IGL). Therefore, this PCD is different from early NOND and is caspase-dependent [78].

Apoptosis of Cerebellar Nuclei Neurons

After treating neonatal rats with ethanol, cerebellar nuclei neurons undergo apoptosis. Three hours after the lesion forms, the axotomy is initiated in cerebellar nuclei and neurodegeneration begins within 48 h. Apoptotic cell morphology has been observed, but during normal development of cerebellar nuclear neurons, apoptosis does not occur [78].

Autophagy and Cerebellum Development

As discussed above, autophagy is a self-degradation lysosomal system that was initially described in single-cell organisms as an adaptation mechanism to nutrient supply fluctuations and to recycle various cellular organelles [83, 124].

The nervous system complex ontogenesis is especially sensitive to dysregulation of autophagy. This is shown by the axonal growth, neural tube defects, and impairment of migration following either inactivation or downregulation of autophagic genes [1, 8, 73]. Autophagy plays essential roles in the late stages of embryonic and postnatal development [21]. Defects in autophagy lead to impairment in the number of neural progenitors and lead to incorrect differentiation and development [1]. For example, an autophagy malfunction causes inappropriate neurotransmitter processing and secretion [1]. There are many neurodegenerative disorders that are caused by defective autophagy mechanisms such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) [61, 62]. The other consequences of defects in autophagy genes such as Atg5 and Atg7 mutant mice include perinatal lethality in suckling kids and neurodegeneration symptoms [61, 62]. In addition, ablation of Atg7 causes dystrophy of Purkinje cell axon terminals in the cerebellar nuclei [21, 82].

Another study on the *ULK1* gene in mammals, which is an orthologue of the yeast *Atg1*, showed its important role in autophagy machinery. ULK1 is a protein kinase that plays a significant role in the early autophagosome formation. When autophagy is induced, ULK1 kinase separates the Ambra1/Beclin-1 complex from the dynein complex, to initiate the autophagy process. During embryogenesis, Ambra1 is expressed in the CNS, specifically in the neural plate. In mice with the *Ambra1* mutant, in which the autophagy machinery is deficient, apoptosis was observed. This result showed the relationship between autophagy, apoptosis, and cell proliferation. Therefore, Ambra1 is an essential protein for the control of cell proliferation during development of the CNS [21]. These data indicate that in the cerebellum, autophagy is involved as a part of PCD in parallel with apoptosis. The autophagic cell death act is an alternative PCD when apoptosis is inhibited in the rat cerebellar granule cell [84]. Both reactive oxygen species (ROS) and autophagy also promote apoptosis in this model [84]. Degeneration of Purkinje cells is a common feature of inherited ataxias in humans and mice. Association of the autophagy

pathway with mitochondria, which is also known as "mitophagy," is reported in Purkinje cell degeneration (*pcd*) mouse. This highlights a link between mitochondrial dysfunction, autophagy, and Purkinje cell degeneration in the cerebellum [14]. In conclusion, autophagy is an important process for survival and development of cerebellar cells [82].

Involvement of UPR in Cerebellum Development

As discussed above, ER stress and UPR participate in many physiological processes such as differentiation and development in different organs and are also involved in pathogenesis of various neurological diseases [42, 94]. The developing brain is highly sensitive to different kinds of environmental stresses (e.g., infectious pathogens, pollutants, alcohol, drugs, and malnutrition), which often cause ER stress [94]. One of the earliest pieces of evidence on the involvement of UPR pathways in brain cells showed that inhibition of global protein synthesis occurs during brain neuron ischemia [48]. In this condition, the UPR can degrade misfolded/unfolded proteins through activation of the ER-associated degradation to ensure a balance of protein-folding capacity that is crucial for cerebellar Purkinje cell survival [47, 69, 128]. Additionally, recent reports suggest that, compared with immature brain neurons, mature neurons are more susceptible to the ER stress and apoptosis induced by tunicamycin. This suggests that the UPR is developmentally involved during neurogenesis [110]. The UPR is generally responsible for the hierarchy and lineage relationships developed in the CNS cells in various animal models [36, 66]. ER stress induction by tunicamycin and thapsigargin induces neuronal differentiation, while the glial differentiation of mouse embryonic stem cells is inhibited via PERK and IRE-1 branches of the UPR [16]. Laguesse and coworkers proposed a model suggesting that dynamic regulation of the UPR pathways is critical to switch from direct to indirect neurogenesis [66]. During cerebral cortex development, the UPR promotes neurogenesis. In the rat cerebellum, development of white matter tracts is dependent on a dramatic increase in membrane protein and lipid production in oligodendrocytes to facilitate myelin production [92]. A substantial peak in ER stress signaling IRE1 and ATF6, but not PERK, as well as the UPR mediators GRP78, GRP94, calreticulin, CHOP, and PDI, has been observed in the developing rat cerebellum [92]. In addition, BiP/GRP78 has a critical function in the development of the cerebellum as well as other neuronal functions. GRP78 knock-in mice show defective layer formation in their cerebral cortex and cerebellum [50]. A few specific ER chaperones can also play a direct role during proliferation and early development of the cerebellum to ensure homeostasis for increased activity of protein secretion [22]. For example, the ER-resident protein ORP150/HSP12A is involved in cerebellum development. Transgenic expression of this protein in neurons reduces Purkinje cells apoptotic cell death and their vulnerability to hypoxic and excitotoxic stress, which subsequently leads to maintaining survival of these cells during

cerebellar development [59]. Defects in BAP (SIL1), another regulator of UPR, can also cause damage in cerebellar Purkinje cells [128, 129] and cerebral ataxia disease [5, 103]. The Marinesco–Sjögren syndrome characterized by cerebellar ataxia is associated with mutation in SIL1 gene [5, 103]. These reports suggest that dynamic regulation of the UPR is needed for balance between proliferation and differentiation in the cerebellum and other tissues [66].

CLCC1, a transmembrane protein in ER, was shown to play an important role in maintenance of ER homeostasis in the young cerebellum. Mutation in this gene results into few pyknotic granule cells in the 3-month-old cerebellum; BIP upregulation and ubiquitin-positive inclusions were observed in these neurons [53].

Conclusion

The role of apoptosis has been investigated in different aspects of development including cerebellar development. Many important roles of apoptosis have been identified in regulation of cerebellar development. Recently, autophagy and the UPR, which are major cellular responses to intra- and extracellular stress, have been shown to also play essential roles in regulation of cerebellar development. All of the mechanisms described in this chapter are tightly interconnected and affect each other. Therefore, future research should consider the regulation of different organ development, including cerebellar development, focusing the regulatory effects of apoptosis, autophagy, and UPR on this process based on their interconnected points. Because apoptosis, autophagy, and the UPR are regulated based on mitochondria, lysosomes, and ER functions, respectively, the future of cerebellar development research will probably change to organelle-based investigations and their role in development. Therefore, developing models that aim to use mis-functional organelles including mitochondria, lysosomes, and the ER will be an asset to significantly increase knowledge in the field of neurodevelopment.

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The Ubiquitin Proteasome System and Cerebellar Developmental Disease

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Abstract A variety of developmental diseases of the cerebellum are associated with dysregulation of proteins regulated by the ubiquitin proteasome system (UPS). Dysfunction of the UPS is observed in several types of spinocerebellar ataxias associated with polyglutamine accumulation. Spinocerebellar ataxia type 3 is caused by a genetic defect ion the Atxn3 gene, which codes for a deubiquitinase enzyme. Defects in expression of a variety of ubiquitin ligases are associated with Friedreich's ataxia, ataxia-telangiectasia, and cerebellar hemangioblastoma. Mutations in a number of genes for ubiquitin ligases are risk factors for autism. Subtypes of medulloblastoma are associated with specific defects in proteasome subunits and with deficiencies in components of the APC/C ubiquitin ligase complex regulating the cell cycle. Targeting various components of the UPS system may contribute to a future therapeutic approach which restores protein homeostasis in various cerebellar diseases.

Abbreviations

AIP	Atrophin-interacting protein
A1UP	Ataxin-1 ubiquitin-like interacting protein
APC/C	Anaphase-promoting complex/cyclosome
ASD	Autism spectrum disorder
AT	Ataxia-telangiectasia
ATM	Ataxia-telangiectasia mutated
ATXN1	Ataxin 1
ATXN3	Ataxin 3
CAG	Cytosine-adenine-guanine repeat
CHFR	Checkpoint with forkhead and ring finger domains
DRPLA	Dentatorubropallidoluysian atrophy

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DUB	Deubiquitinase
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
E1	Ubiquitin-activating enzyme
FRDA	Friedreich's ataxia
FXN	Frataxin
HIF-1	Hypoxia-inducible factor 1
ITPR	Inositol triphosphate receptor isoform
MB	Medulloblastoma
MJD	Machado-Joseph disease
RNF	Ring finger protein
SCA	Spinocerebellar ataxia
UBR	Ubiquitin protein ligase E3 component N-recognin 4
UPS	Ubiquitin proteasome system
USP	Ubiquitin-specific protease
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau protein

Introduction

In this chapter, we will discuss cerebellar diseases from the perspective of the ubiquitin proteasome system. In some of these diseases, the ubiquitin proteasome system (UPS) plays a key role in the disease, while in others, the role of the ubiquitin proteasome system, if any, is not clear. In at least three types of spinocerebellar ataxias, the protein product of the gene associated with the disease is an E3 ubiquitin ligase. We also discuss the role of the ubiquitin proteasome system in cerebellar hemangioblastoma, in autism, and in medulloblastomas in terms of deficiencies of the ubiquitin proteasome system.

The Ubiquitin Proteasome System

The stability of most cellular proteins is controlled by the rate of their degradation through the proteasome, a catalytic chamber. Prior to degradation, the proteins are tagged with the ubiquitin molecule via a series of enzymes, a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) [1]. An additional enzyme, a deubiquitinase (Dub), functions to remove ubiquitin [2–4]. This provides a way of recycling ubiquitin. Deubiquitinases can also, together with specific ubiquitin ligases, serve as on/off switch mechanism for rapidly controlling proteins that are required for a short, or defined, period of time. The subunits and assembly of the proteasome has recently been described in detail [5]. Herein, we discuss the role of the ubiquitin proteasome system in various developmental diseases of the cerebellum.

Ataxia and Spinocerebellar Ataxia

Ataxia is a neurological condition in which lack of coordination of muscle groups leads to abnormal gait. Such neurological conditions are often associated with degeneration of parts of the cerebellum and degeneration of neuronal pathways between the cerebellum and spinal cord; hence, they are called spinocerebellar ataxias (SCAs). As genes for various SCAs were identified, they were sequentially numbered. Currently there are over 40 subtypes of SCAs identified. SCA type 41, for example, is associated with a mutation in the TRPC3 gene [6]. A mouse model for this disease, the moonwalker mouse, has a mutation of this gene [7]. A number of SCAs are associated with defects in the ubiquitin proteasome system. Tarlac and Storey [8] have noted that proteasome components and ubiquitin are often found co-localized with abnormal aggregates of proteins in neurons of SCA patients, particularly those with polyglutamine diseases.

Spinocerebellar Ataxia Type 1 (SCA1)

SCA1 is a polyglutamine disease [9]. It is associated with a CAG (cytosine-adenine-guanine) repeat in the ataxin 1 gene (*ATXN1*) [10]. Loss of ATXN1 function is reported to contribute to the pathogenesis of SCA1 [11]. One protein to which the ataxin 1 protein binds is ubiquilin 4 (aka ataxin-1 ubiquitin-like interacting protein, A1UP) [12]. This protein also interacts with subunits of the proteasome, contributing to the mechanism by which misfolded proteins are degraded in this structure [13]. The E3 ubiquitin ligase CHIP can ubiquitinate wild-type ataxin 1, as well as its expanded polyQ form, and can protect against the toxicity of the expanded ataxin-1 protein [14]. Enhancing CHIP activity has been proposed as therapy for polyQ diseases [15].

In a mouse model of SCA1 gene expression in the cerebellum of the ATXN1, polyQ mice were compared to that of wild type and to that of ATXN1 knockout mice [11]. These investigators analyzed the genes altered in the strains of mice by Kegg analysis. Two sets of genes were expressed in opposite directions in ATXN1 knockout and ATN1 knockin mice, genes that could provide information concerning the mechanism of SCA1 pathogenesis. The two sets of genes, according to the Kegg analysis, were a group including three genes of the TCA cycle and a second group of five genes associated with the ubiquitin-mediated proteolysis (see their supplemental Table 2). The five ubiquitin ligase genes in this table were ANAPC2, UBE2O, UBE3B, WWP2, and MID1 (aka Trim18). It should be noted that WWP2 is also known as atrophin-interacting protein 2 (AIP2), (see below). Mutation of TRIM18 may result in a variety of genetic defects, including the Dandy-Walker malformation [16] and, in some patients, agenesis of the cerebellar vermis [17].

Spinocerebellar Ataxia Type 2 (SCA2)

SCA2 is another polyglutamine disease. It is caused by a mutation in the ATXN2 gene [18, 19]. Mutation of this gene can also result in a Parkinson-like syndrome, as well as in amyotrophic lateral sclerosis [20]. Although the ubiquitin and ubiquitin-like conjugation database (UUCO) classifies the ATXN2 protein as an E3 ligase of the ring family, most publications on SCA2 have not noted this.

Spinocerebellar Ataxia Type 3 (SCA3)/Machado-Joseph Disease and Ataxin 3

Machado-Joseph disease (MJD), although rare, is one of the most common of the spinocerebellar diseases. It was named after two individuals in which it was first described [21]. MJD is also referred to as spinocerebellar ataxia type 3 (SCA3); however, early investigators distinguished the two [22]. It is a progressive neurodegenerative disease leading to paralysis and death [23]. In addition to ataxia, the symptoms of this disease included memory deficits, dysarthria, alterations in saccadic eye movements, and dysphagia [24]. There is no current cure for this disease. SCA3/MJD, an autosomal dominant disease, is associated with a genetic abnormality (CAG trinucleotide repeats) of the *ATXN3* (ataxin 3) gene [25–28], a gene located on chromosome 14 (at 14q32.12). The *Atxn3* gene codes for the protein ataxin 3. In SCA3/MJD, ataxin 3 accumulates in neurons as the disease progresses [29]. SCA3/MJD is one of a number of polyglutamine (polyQ, caused by expanded cytosine-adenine-guanine (CAG) repeats) neurodegenerative diseases associated with protein aggregates in neurons [30, 31]. The components of the ataxin-3 protein, including the polyQ region have been described and illustrated by Matos et al. [28].

Ataxin 3 has been identified as a deubiquitinase enzyme [28]. It has several ubiquitin interacting regions which account for its binding to polyubiquitinated protein chains [28]. Riess et al. [27] have illustrated a model of the normal function of ataxin 3. In this model, ataxin 3 facilitates the transport of ubiquitinated proteins to the proteasome for degradation. In SCA3/MJD, ubiquitinated proteins accumulate and proteasome activity is inhibited [32]. There is some data suggesting that in end-stage SCA3/MJD, there is a defect preventing assembly of the two major components of the proteasome, the proteolytic component and the regulatory component [33]. The presence of ubiquitin in neuronal inclusions in polyQ diseases has been taken as evidence for a role of the ubiquitin proteasome system in the pathogenesis of these disorders [32].

Ataxin 3 functions as a polyubiquitin-editing enzyme rather than simply as an enzyme which completely deubiquitinates its substrate [28, 34, 35]. According to Windborn et al., it binds to both Lys [36] and Lys [37] ubiquitin linkages but preferentially cleaves Lys [37] linkages [35].

In SCA3/MJD, the soluble polyglutamine proteins are toxic [28] and probably interfere with the normal function of ataxin 3 as a deubiquitinase. Ataxin 3 has been

shown to interact with several ubiquitin ligases including CHIP and Parkin [28, 38]. It regulates the activity of these ligases by removing ubiquitin from them. It has been suggested that the activity of Ataxin 3 is itself enhanced by ubiquitination [39, 40]. Its cellular role has been related to protein quality control [40]. Chai et al. [41] showed that the proteasome suppresses polyglutamine aggregation in neurons of SCA3/MJD patients and suggested that the ubiquitin proteasome pathway has a key role in polyglutamine diseases including SCA3/MJD. However, data on the precise role of the proteasome, or its subunits, in SCA3/MJD is lacking. Rat and mouse models of SCA3/MJD have been developed [42, 43]. These models will contribute to determining the role of ataxin 3 and its polyglutamine form in development and treatment of SCA3/MJD.

Spinocerebellar Ataxia 5 (SCA5)

Mutations in the *SPTBN2* gene reportedly cause SCA5 [44]. This gene codes for one of the spectrin proteins, B-III-spectrin. Spectrin is an F-actin crosslinking protein composed of two chains, alpha and beta, making up a helix. It has a mechanical role in maintaining the shape of the cell but is also involved in cell signaling [45]. The alpha chain is reported to have E2 ubiquitin conjugase activity as well as E3 ubiquitin ligase activity [46–48]. The role of the alpha chain E2/E3 activity in the development of SCA5 has not been studied.

Spinocerebellar Ataxia 6 (SCA6)

SCA6 is a rare cerebellar ataxia with additional oculomotor symptoms. Both SCA6, which is progressive, and an episodic non-progressive ataxia, subtype 2 (see below), are associated with a mutation in the calcium channel subunit gene, *CACNA1A*. SCA6 is another polyglutamine disease caused by CAG repeats in the gene [49]. CACNA1A, also known as SCA6, is associated with the E3 ubiquitin ligase BCL6 [36]. This association is not well characterized.

Spinocerebellar Ataxia 7 (SCA7)

SCA7 is another disease caused by CAG nucleotide repeats. It is caused by a mutation in a gene on Chromosome 3, *Atxn7*. It is a progressive disease that results in ataxia and blindness. The ataxin-7 protein is part of a protein complex, the SAGA complex that acts as a DUB which regulates transcription of a number of genes [50, 51]. The DUB protein which contributes to this complex is USP22 [52]. The polyglutamine expansion of the ataxin-7 protein apparently interferes with the function of the USP22 as a gene silencer [52, 53].

Spinocerebellar Ataxia 8 (SCA8)

SCA8 is associated with a trinucleotide repeat expansion in the two overlapping genes, *ATXN8* and *ATXN8OS* [54]. The latter codes for an antisense RNA for the ubiquitin ligase KLHL1 [55]. Both genes are highly expressed in the cerebellum and other brain tissues.

Spinocerebellar Ataxia 15 (SCA15)

SCA15 is a cerebellar ataxia in which atrophy of parts of the vermis is reported [56]. Mutations of the IPTR1 gene are associated with this disorder. Mutations of this gene are associated with abnormal regulation of calcium release by calmodulin and UBR4 (aka p600), a ubiquitin E3 ligase [57]. Mutations of UBR4 are associated with at least one subtype of episodic ataxias (see below).

Spinocerebellar Ataxia 17 (SCA17)

Sca17 is caused by a mutation in the gene (*TBP*) for the Tata-box-binding protein. In a model of Sermwittayawong and Tan [58], the SAGA complex interacts with TBP to regulate transcription. These investigators, however, did not discuss the deubiquitinase activity of the SAGA complex in their model. If deubiquitinase activity is generally associated with the SAGA complex, as suggested by others [59, 60], it may also be important in regulating TBP in the cerebellum.

Spinocerebellar Ataxia Type 19 (SCA19) and Type 22 (SCA22)

SCA19 and SCA22 have been associated with mutations in gene for a potassium channel component, KCND3 [61]. This protein has been classified as an E3 ubiquitin ligase of the BTB family in a supplemental table of a recent publication [62].

CHIP and Gordon Holmes Syndrome

It has been shown that mutations in the *Stub1* gene, which codes for the ubiquitin ligase CHIP, are associated with a number of autosomal recessive cerebellar ataxias [37, 63]. In Gordon Holmes Syndrome (ataxia associated with hypogonadism), mutation in the *Stub1* gene and loss of CHIP has been identified as the probable

cause of this disorder [64]. Ronnebaum et al. [65] concluded that CHIP is required for maintenance of normal cerebellar function.

Dentatorubropallidoluysian Atrophy

Dentatorubropallidoluysian atrophy (DRPLA) is another autosomal dominant neurodegenerative disease associated with cerebellar ataxia [66, 67]. It is also referred to Naito-Oyanagi disease [68]. Like SCA3/MJD, it is a genetic abnormality with trinucleotide repeats and polyglutamine proteins [69–71]. In DRPLA, there is an abnormality of the atrophin-1 gene (*Atn1*), expansion of a CAG repeat [72]. The abnormal form of the atrophin-1 protein accumulates in the brains of DRPLA patients [72].

Several atrophin-interacting proteins including AIP1, AIP2, AIP3, AIP4, and AIP5 have been identified [73]. Three of them are E3 ubiquitin ligases. AIP2 is also known as WWP2 (WW domain containing protein ligase 2). AIP4 has been identified as a ubiquitin ligase homologous to the mouse E3 ligase Itch [74]. Among the substrates of this E3 ligase are the proteins Notch [75] and JunB [76]. AIP5 is also known as WWP1 (WW domain containing protein ligase 1). AIP1 and AIP3 have not been described as E3 ligases. They are membrane-bound proteins with guanylate kinase-like regions [73].

Friedreich's Ataxia and Ubiquitin-Competing Molecules

Friedreich's ataxia (FRDA) is a hereditary protein disease. It is inherited as an autosomal recessive disease that initially presents itself in symptoms of gait disturbance and lack of coordination. FRDA is a disease that progressively impairs the muscular system. Other systems involved may include vision, hearing, speech, carbohydrate metabolism, and cardiac disorders. The pathology of FRDA has been reviewed by Koeppen AH 2011 [77]. FRDA results from failed transcription of the frataxin (*FXN*) gene [78, 79]. Gene silencing may contribute to this failure in transcription [77]. A deficiency in the FXN protein leads to the degenerative conditions characteristic of FRDA [80]. FXN has been located to the mitochondrial matrix. It is thought to play a significant role in maintaining adequate levels of iron in mitochondria [81].

Currently, there is no effective treatment for FRDA. However the FRDA phenotype, in an in vitro mouse model, was partially reversed, using viral vectors encoding for the *FXN* gene [82]. This model provided an incentive to use this approach in humans. In humans, efforts have been made to reactivate the *FXN* gene using nicotinamide [83]. Underlying this research effort is the view that epigenetic regulation of the *FXN* gene is possible.

Another approach to increasing FXN is to manipulate its degradation. FXN is a protein that is degraded by the ubiquitin proteasome system [84]. Theoretically, proteasome inhibitors could be used to increase tissue concentrations of FXN. However this approach is limited to those inhibitors that cross the blood brain barrier. Another limitation is that proteasome inhibitors are not specific enough. Rufini and colleagues have identified and tested a series of lead compounds capable of interfering with FXN ubiquitination and degradation [84]. Recently, they found that small molecules which bind to FXN compete with ubiquitin for binding to FXN (at a specific site on the molecule, lysine 147) and lead to accumulation of FXN [85]. They named these molecules ubiquitin-competing molecules. Their results provided a rationale for a therapeutic use of ubiquitin-competing molecules in FRDA disease.

Episodic Ataxia and Ubiquitin Ligases

There are currently eight separate clinically recognized episodic ataxias (EA) [86]. In one form of EA, subtype 8, the UBR4 (ubiquitin protein ligase E3 component N-recognin 4) gene on chromosome 1 was reported as the likely source of genetic variations causing this ataxia [57]. UBR4 (aka p600) is a ubiquitin E3 ligase [87, 88] that interacts with calmodulin, a calcium-binding protein. UBR4 also binds to ITPR1 (inositol trisphosphate receptor isoform 1), which regulates calcium release from the endoplasmic reticulum [57]. Conroy et al. [57] suggested the hypothesis that interference with normal binding of calmodulin and/or ITPR1 to UBR4 resulted in a dysfunctional calcium-sensing system leading to ataxia.

One of the most common types of EA (subtype 1) is reportedly caused by variations in a gene *KCNA1*, which codes for a potassium channel protein [89]. KCNA1 has been recently identified as having E3 ubiquitin ligase activity [62] (see supplemental Table 4 in this reference). As noted above, EA subtype 2 is caused by mutation in the calcium channel subunit gene, *CACNA1A*.

Ataxia Telangiectasia (AT) and the ATM Protein

Ataxia telangiectasia (AT), also known as Louis-Bar's syndrome [90] is an autosomal recessive disorder that results in various clinical symptoms including progressive ataxia. AT patients have a defect in a gene associated with the repair response to double-strand DNA breaks resulting from oxidative stress [91]. The ATM (ataxia telangiectasia mutated) protein, a serine-threonine protein kinase, phosphorylates several enzymes necessary for activation of the DNA damage checkpoint and repair response after double-strand DNA breaks [92].

Other proteins involved in ATM activation include the ubiquitin ligases RNF8 (ring finger 8) and CHFR (checkpoint with forkhead and ring finger domains) [93]. Via phosphorylation, ATM can activate or inactivate many different proteins. Its effect on the ubiquitin proteasome system during activation of the response to double-strand DNA breaks has been described by Shiloh and Ziv [93] as having several phases: (1) recruitment of ATM to the site of double-strand breaks (partially mediated by the E3 ubiquitin ligase SKP2), (2) a kinase cascade stimulating the phosphorylation of many proteins including other kinases, (3) recruitment of proteasomes to the site of DNA damage [94], (4) modulation of ubiquitin ligases and DUBs (deubiquitinases) by phosphorylation, and (5) phosphorylation of substrates of E3 ligases preparing them for ubiquitination. Thus E3 ligases control the stability of proteins such as p53 and NFκB. Among the E3 ligases listed by Shiloh and Ziv as influenced by ATM include Cop1 (aka RFWD2), MDM2, MDMX, and SIAH1. The deubiquitinase USP10 is also phosphorylated by ATM. Thus it can be safely concluded that the ubiquitin proteasome system plays an important role in the ATM response. Eventually this information may be used to design therapeutic molecules that can be used in the management of AT.

Cerebellar Hemangioblastoma and the von Hippel-Lindau Protein

Hemangioblastomas of the cerebellum are frequently associated with von Hippel-Lindau (VHL) disease [95, 96]. In this disease, there is a deficiency in the gene for the VHL tumor suppressor protein and overexpression of VEGF (vascular endothelial growth factor) [96]. Hemangioblastomas probably originate from hemangioblast progenitor cells [97].

The molecular mechanisms by which loss of the VHL gene or VHL protein leads to susceptibility to hemangioblastoma has been described [98]. The VHL protein has been shown to be a ubiquitin ligase [99]. One of its substrates is the transcription factor HIF-1α [100], a transcription factor for a number of proteins including VEGF [101]. Under normal conditions (normoxia), HIF-1α is ubiquitinated by VHL and degraded by the proteasome [102].

Autism-Associated Genes

The development of the cerebellum has been shown to differ in autistic patients compared to controls [103]. MRI studies showed hypoplasia of the cerebellum in autistic patients [104]. Postmortem studies showed significantly decreased numbers of Purkinje neurons in the cerebellum of patients with autism spectrum disorders (ASD) [103]. Among the genes associated with ASD is the gene for the ubiquitin

ligase UBE3A [105]. It was reported as upregulated in cells from individuals with autism [106]. The *UBE3A* gene is better known as the gene which when deficient causes Angelman syndrome [107]. It is a maternally expressed gene. The protein encoded by this gene is the E6-AP protein [108]. It is named for its association with the papillomavirus protein E6.

Recently Louros and Osterweil [105] have noted that mutations in a number of genes of the ubiquitin proteasome system have been identified as risk factors for ASD. In addition to *UBE3A,* ten other ubiquitin ligases were documented as risk factors for ASD (UBE3B, UBE3C, PARK2, FBXO40, RFWD2, Cullin 3, Cullin 7, HECW2, HERC2, and HUWE) in this review. The genes coding three deubiquitinases (USP9Y, USP45, and USP7) and the gene for the proteasome subunit PSMD10 were also listed as risk factors. The authors point out that these data provide strong evidence for dysregulation of protein degradation in ASD. The number of ubiquitin proteasome proteins listed as risk factors may reflect the heterogeneity of the ASD diseases.

Medulloblastoma and Ubiquitin Proteasome Components

Medulloblastoma, described as a malignancy of the cerebellum, actually describes a group of heterogeneous tumors, differing in histology, genetic expression, clinical outcome, and response to treatment. A consensus classification, however, was reported in 2012 [109]. In this classification, four major subtypes of medulloblastoma were recognized, the WNT group, the SHH group, and two additional groups simply referred to as groups 3 and 4. In 2015, we suggested the possibility of classification of MBs according to their expression of ubiquitin ligases [110]. In support of this view are supplemental data of Thompson et al. [111] showing differential expression of at least 50 ubiquitin ligases among the various subtypes of MB. Since the Thompson data were reported before 2012, these investigators recognized five subgroups of MB rather than the four subgroups of the consensus classification. In a recent review [112], we identified these E3 ligases and indicate whether they were significantly upregulated or downregulated in the various subgroups of the Thompson supplementary dataset. We also noted differential expression of 12 deubiquitinases among the various subtypes of MB in the Thompson dataset. Since the publication of our review, we noted that expression of the gene *UBE3A*, also an E3 ligase, was also differentially expressed among some of the Thompson MB subgroups. We have noted above that the *UBE3A* gene is associated with Angelman syndrome and autism as well. Thus, in addition, this ubiquitin E3 ligase could be useful as a marker gene for a subtype of MB, the Thompson Group E MB (equivalent to MB consensus subtype 3). We noted above that the *UBE3A* gene codes for a protein, E6-AP. On further examination of the Thompson dataset, we note that several ubiquitin-conjugating enzymes are differently expressed among the various

Table 1 Ubiquitin-conjugating enzymes in MD groups of Thompson et al.

Gene	Location	Group A	Group B	Group C	Group D	Group E
UBE2B	5q31.1			Up	Down	Down
UBE2C	20q13.12			Down	Up	
UBE2D2	5q31.2			Down		Up
UBE2E1	3p24.2					Down
UBE2N	12q22	Up			Down	
UBE2V1	20q13.3		Down	Down	Up	Up
UBE3A	15q11.2	Up				Down
UBE2K	4p14			Up		Down

MB subtypes. In Table 1 below, we list the ubiquitin conjugases that were significantly upregulated or downregulated in the Thompson dataset compared to the other MB groups. Thus E2 conjugases and E3 ligases and deubiquitinases could all be useful as marker genes for the various subtypes of MB.

Another remarkable feature of the Thompson dataset [111] is that it shows differential expression of genes for proteasome subunits among the different subtypes of MB. The Wnt subgroup showed significant depression of expression of the *PSMB1 gene*; the SHH subgroup of MBs showed significant depression of expression of seven separate genes for proteasome subunits; the Thompson dataset also showed that their Group A MBs had significantly increased expression of genes for 13 separate proteasome subunits, including the genes for two catalytic subunits. The genes for eight subunits of the proteasome, including two catalytic subunits, were significantly decreased in their group C tumors. The final group of Thompson MBs, group E, also showed significant variations in several proteasome subunits. This was illustrated in the review of Vriend and Marzban [112] and reproduced as Fig. 1 (by permission). These results showed that genes for proteasome subunits are "signature" genes for subtypes of MBs and raise the possibility of targeting proteasome subunits therapeutically.

One ubiquitin ligase complex suggested in a therapeutic context for MB is casein kinase 1 delta, a substrate of the APC/C (anaphase-promoting complex/cyclosome) complex [113]. The APC/C ubiquitin ligase is an important regulator of mitosis. Among the factors that regulate its activity is the human cytomegalovirus [114]. Many medulloblastomas are reportedly infected with this virus [115, 116]. Although a causative relationship between cytomegalovirus and MBs has not been definitively established, Baryawno et al. [116] have suggested an important role for this virus in the development of MB. This virus may be more significant in subgroups of MBs in which the activity of the APC/C ubiquitin ligase complex is impaired than in MBs in which this complex is fully functional. Further investigation on the interaction of viruses and ubiquitin ligases may provide information leading to new therapeutic approaches for several cerebellar diseases.

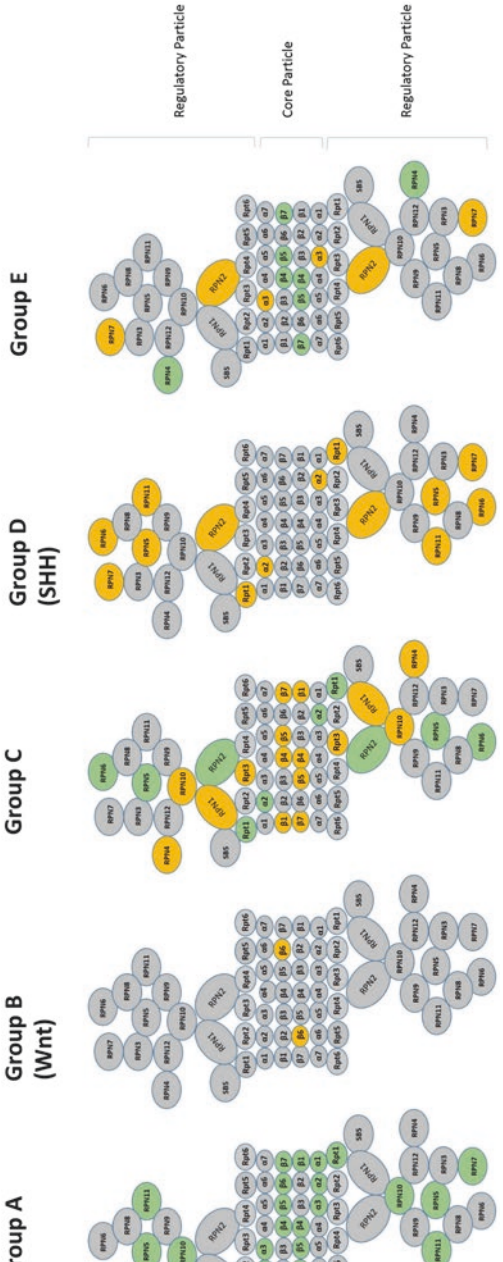

Fig. 1 Differential expression of proteasome subunits in MB subtypes in Thompson supplemental dataset (used with permission, Springer, Cellular and Molecular Life Sciences, 2016). *Green* significantly increased. *Orange* significantly decreased compared to other groups. *Gray* not in dataset or not significant

Prospective Expectations

Various developmental diseases of the cerebellum are associated with abnormal protein regulation [8]. It is becoming clear that a dysfunctional UPS has a key role in many of these disorders. As subsequent research identifies the specific components of the UPS that are dysfunctional, the opportunities arise to target these constituents therapeutically. Inhibitors of ubiquitin ligases and ubiquitin conjugases, as well as inhibitors of deubiquitinases, may all be therapeutically significant in the treatment of some of these diseases. In cases in which disease is associated with proteasome dysfunction, proteasome inhibitors or proteasome stimulating proteins may be clinically practical.

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Epigenetics and Cerebellar Neurodevelopmental Disorders

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Abstract Epigenetic mechanisms regulate cellular identity and organ morphology *via* controlling the gene expression program of specific cell types. Such mechanisms are not directly controlled by genomic DNA sequences and can be largely influenced by environmental factors. Epigenetic mechanisms include modification of DNA and DNA-bound proteins (histones), action of large and short regulatory RNA molecules, cross talk between DNA and histone marks, nucleosome positioning, chromatin removdeling, enhancer-promoter interactions, as well as three-dimensional chromatin structure that is in part controlled by global regulators and insulator proteins. Research on epigenetic mechanisms is an emerging hot topic today that may very well be due to the potential reversibility of epigenetic marks. Such characteristics of epigenetic modifications have brought them into the front row of research for cutting-edge therapeutic strategies. The challenge would be of course the very large number of genes that will be targeted by most epigenetic drugs that are capable of global modulation of epigenetic marks and a purposeful management of selectively targeting disease-associated genes in balance with global effects of these drugs.

Like all parts of our body, development of the central nervous system and the brain is regulated through epigenetic mechanisms. It is not of surprise that deregulation of epigenetic modifications may lead to human disease and neurodevelopmental disorders. In this book chapter, I will focus on main epigenetic mechanisms that control the brain and cerebellum development. I will then discuss some of the common neurodevelopmental disorders that have proven epigenetic components that provide important insight toward the future research on epigenetics and cerebellar neurodevelopmental disorders.

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Introduction

Genetic material of eukaryotic cells exists as double-stranded DNA molecules packed around an octamer group of DNA-bound proteins (histones), making up the core structure of "nucleosomes" known as the fundamental units of the "chromatin" structure. The term chromatin was first described by Flemming and Zelltheilung in 1882, as they referred chromatin to the "densely stained nuclear DNA" [1]. At the time, the basic structural organization of DNA molecules was unknown, and it was not until seven decades later and in 1953, when Watson and Crick discovered the double helix DNA structure [2]. "Epigenetics" is yet another term that was described by Conrad Waddington in 1942, also prior to the discovery of DNA structure. Waddington referred to "epigenetics" as the "casual interactions between genes and their products which bring the phenotype into being" [3]. Since then, our knowledge on epigenetic regulatory mechanisms and the associated epigenetic molecular modifications has grown substantially with an impressive >52,000 research and review articles in this area of research thus far.

In this book chapter, I will describe three major epigenetic mechanisms that include DNA methylation, histone post-translational (PTM) modifications, and regulatory RNA molecules. I will discuss the main epigenetic players in establishing the "epigenetic code" that include "writers," "readers," and "eraser" of DNA methylation and histone post-translational modifications, with limited discussion of the cross talk between these two types of epigenetic modifications. I will briefly overview other types of epigenetic mechanisms such as unidirectional chromatin remodeling and bivalent marks at the developmentally important *Hox* genes. Lastly, I will discuss some examples of neurological brain disorders with an epigenetic or epigenetic-genetic basis that will provide some thoughts on the future direction of this line of research.

Epigenetics

In 1942, Conrad Waddington used the word "epigenetics" in an attempt to explain how during development the "genotype" of an organism directs its individual cell morphology throughout life and dictates its "phenotype" [3]. The term "epigenetics" is primarily rooted from the Greek word of "epi," meaning "above" or "on" the "genetics." In general, epigenetic control begins as early as the life begins, instructing the cellular fate commitment from the very first cellular cleavage, and continues through embryonic development, and after birth during infancy, childhood, adulthood, and throughout life [4]. By regulating the gene expression program of individual cells, epigenetic information determine and dictate the readout of the genetic material, so that despite sharing the same genomic DNA in all somatic cells, distinct morphologies, identities, and functions of different cell types of our body are established [5]. It is of significant importance that such mechanisms are greatly impacted by environmental factors, one example being the negative influence of maternal

in utero exposure to alcohol during embryonic development that causes neurological disorders, and mal-nutrition or stress after birth with negative impact in the body and the brain. Accordingly, the environmental factors are capable of manipulating and re-organizing the composition of epigenetic marks on DNA molecules leading to a different outcome in cellular gene expression program exhibiting neurological consequences, i.e., fetal alcohol spectrum disorders (FASD) in the case of maternal alcohol exposure. While we cannot deny a possible contribution of genetic susceptibility for FASD, without maternal exposure of a developing embryo to alcohol, there will not be any FASD development in a child. Collectively, this will highlight the involvement of environmental factors in human disease and neurological disorders.

DNA Methylation

Perhaps the very the first evidence of epigenetic modifications goes back to 1963, when DNA and RNA methylation was primarily noticed [6]. However, the discovery of eukaryotic DNA methylation happened 14 years later and through the research of Razin and Cedar in 1977 [7]. Today, there are over 57,000 published original research and review articles on DNA methylation, capturing the impact of these discoveries over the last four to five decades. As research progresses, new technologies are developed for genome-wide DNA methylation studies, and new terms are introduced in this field that refer to different types of global studies. These terms include "methylome" that captures all different types of genomic DNA modifications, "methylomics" that refers to studies aiming to characterize the cross talk of "histone code" and DNA methylation, and lastly "gethylome/gethylomics" that connects "methylome/methylomics" to "genome/genomics" [8–10]. Research by independent groups has highlighted the biological importance of DNA methylation during embryonic development, X-chromosome inactivation, genomic imprinting, regulation of gene expression, alternative splicing, and stem cell differentiation, among other regulatory mechanisms [11]. As expected, deregulation of these epigenetic mechanisms and/or mutation in the components of epigenetic machinery may lead to human disease, cancer, and neurological disorders.

Chemically, DNA methylation is characterized by the covalent binding of a methyl group (CH3) to the fifth carbon of a cytosine nucleotide that is usually in the order of "CpG" dinucleotides and is called 5-methylcytosine (5-mC) (Fig. 1). The 5-mC modification is known as the fifth base of genomic DNA and is commonly associated with gene inactivation. Recent studies have further discovered the importance of yet a new form of "non-CpG" methylation in the context of "CpH" methylation, where H can be either A, C, or T [8, 13]. The CpH methylation is relatively abundant in the brain and in neurons, but still much below the rate that CpG methylation occurs, such as in adult mice brain neurons, the ratio of CpG methylation is about ~75%, while CpH methylation is ~25% [14].

In 2009, independent research groups reported a new type of DNA methylation known as 5-hydroxymethylcytosine (5-hmC) [15, 16], which is now referred to as

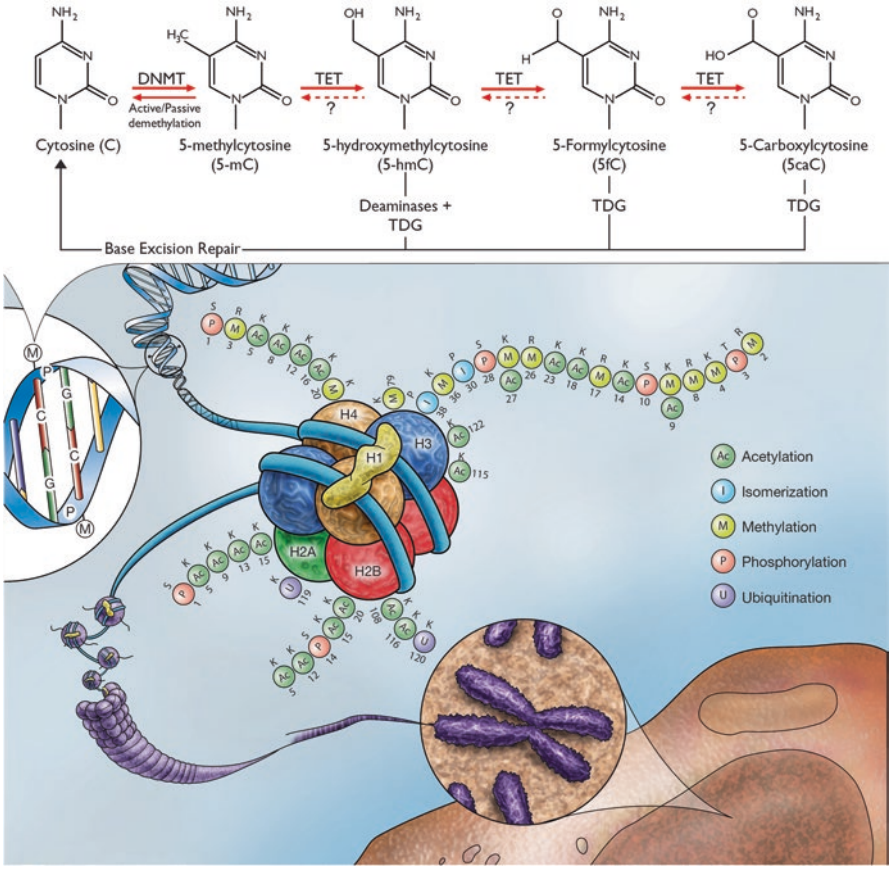

Fig. 1 The basic structure of a nucleosome, different types of DNA methylation, and diverse forms of histone post-translational modifications (*PTM*) are shown. The histone octamer of 2× H2A-H2B, two molecules of histone H3, and two molecules of histone H4 are shown with the double-stranded DNA molecules and histone H1 that connects the adjacent nucleosomes. Different types of histone PTM are shown, along with the DNA methylation at the CpG dinucleotides. At the top, the formation of different types of DNA methylation through the action of DNMT and TET proteins is also shown (Figure is adapted, updated, and modified from Rastegar and Barber 2010 [12])

the sixth base of the genome [17, 18]. The newly identified 5-hmC is highly enriched in embryonic stem cells and Purkinje cells of the cerebellum [15, 16]. Unlike 5-mC, this new form of DNA methylation (5-hmC) is considered to be a hallmark of active genes due to its association with active promoters and presence at the enhancers and genomic sequences of actively transcribed genes downstream of the transcription initiation site(s) [19]. Continued research in this field has led to the discovery of yet other new forms of DNA methylation produced by further oxidization of 5-hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Fig. 1), but our current knowledge about their functional role is limited [20].

DNA Methyl Writers

Members of DNA methyltransferase (DNMT) family are responsible for the deposition of methyl CH3 modification on cytosine nucleotides to establish the formation of 5-mC DNA methylation mark. In mammals, maintenance of 5-mC DNA methylation during replication is the result of DNMT1 enzymatic activity, while DNMT3A and DNMT3B carry out de novo DNA methylation [21]. DNMT3A and DNMT3B establish the primary framework of CpG DNA methylation [22, 23], without favoring the hemimethylated versus unmethylated DNA. The third member of this group is called DNMT3-like protein (DNMT3L) and is considered to be an enzymatically inactive member [23]. In mice, transgenic *Dnmt1* deficiency causes widespread genomic DNA demethylation that leads to embryonic lethality rapidly after gastrulation and during early embryonic development [24]. Similar to DNMT1, both DNMT3A and DNMT3B are essential for proper embryonic development and survival beyond birth. Accordingly, *Dnmt3B*-deficiency in mice leads to embryonic lethality, while *Dnmt3A* knockout mice complete the embryonic development program. However, *Dnmt3A*-deficient mice die shortly after birth, further highlighting the biological importance of DNA methylation [25]. In contrast to these two members of DNMT3 group, mice with *Dnmt3L*-deficiency survive till adulthood, despite the fact that male knockout mice are infertile as their sperms do not mature [26].

In humans, *DNMT1* mutation is associated with neurodegenerative disorders that are autosomal dominant, namely, the "hereditary sensory and autonomic neuropathy with dementia and hearing loss type 1E (HSN1E)" and "autosomal dominant cerebellar ataxia with deafness and narcolepsy (ADCA-DN)" [27, 28]. *DNMT3A* mutation is associated with overgrowth disorders [29], and *DNMT3B* mutations are connected to ICF (Immunodeficiency, Centromere instability, Facial abnormalities) Syndrome that is a rare autosomal disease [30].

DNA Methyl Readers and MeCP2

Once 5-mC DNA methylation is established, this epigenetic modification is recognized, being bound to, and interpreted by the family members of the methyl-binding proteins (MBP). MBP family members consist of MBP1, MBP2, MBP3, MBP4, methyl CpG-binding protein 2 (MeCP2), and Kaiso family proteins. Perhaps the most-studied MBP member is MeCP2, which is also the prototype member of this group discovered by Adrian Bird and his team in 1992 [31].

By binding to 5-mC, MeCP2 represses its downstream target genes *via* multiple mechanisms [32]. The 5-mC commonly marks inactive genes, critically important for transcriptional silencing, imprinting, X-chromosome inactivation, genomic stability, embryonic development, and proper function of the brain [5, 33]. In vivo studies in mouse brain show that MeCP2 specifically binds to 5-mC at the genes that

carry DNA methylation [34]. In vitro DNA-protein binding assays show that MeCP2 preferentially binds to methylated DNA and has low affinity for unmodified DNA. The high-affinity binding of MeCP2 to 5-mC requires MeCP2 N-terminus regions and its methyl-binding domain (MBD) [35]. MeCP2 DNA-binding activities are essential for the proper chromatin structure formation in neurons, where the protein is exceptionally abundant [32]. It is suggested that in neurons, MeCP2 may act more as a global governor of chromatin architecture rather than being a site-specific gene regulator [34]. However, in the absence of MeCP2, a few critical target genes (such as *Bdnf*) are always and specifically altered in a cell-type and brain region-specific manner [36–38], highlighting the role of MeCP2 also as a target-specific transcriptional regulator.

Adding a new layer of complexity to MeCP2 function is the discovery of MeCP2 being capable of binding to 5-hmC in the brain. As stated earlier, the 5-hmC is an abundant DNA modification in the brain and is suggested to mark active genes [39]. This is in direct contrast to 5-mC that usually marks repressed and inactive genes [5]. MeCP2 binding to both 5-mC and 5-hmC is important for its proper function and is mainly mediated through its MBD. Within the MBD mutations, MeCP2 R133C mutation only loses the binding to 5-hmC and not 5-mC; however MeCP2 D121G mutation only inhibits its 5-mC-binding without affecting MeCP2 binding to 5-hmC [39]. In addition to be a transcriptional repressor, MeCP2 is also reported to act as an activator of transcription [40]. Accordingly, it is suggested that MeCP2 acts as a repressor when bound to 5-mC and as an activator when bound to 5-hmC [39].

MeCP2 DNA-binding activities might be more complex than originally thought, due to the presence of two MeCP2 variants (isoforms) that may not be fully redundant in their DNA-binding activities and functional properties. In both mice and humans, *Mecp2/MECP2* gene creates two protein isoforms, MeCP2E1 (also named MeCP2B or MeCP2α) and MeCP2E2 (also named MeCP2A or MeCP2β) with unique N-terminal sequences. These isoforms are produced through alternative splicing of the second exon [41]. In the brain, distinct transcript expression patterns are detected for individual isoforms [42], with *MECP2E1* displaying 10× higher expression. The difference at the N-terminal sequences of MeCP2 isoforms is rather short, with 21 amino acids exclusive to MeCP2E1 encoded by exon 1, and nine amino acids only present in MeCP2E2 encoded by exon 2. The regulation and functional properties of the two MeCP2 isoforms have been the subject of my research over the last decade. By generating isoform-specific antibodies, my lab reported that MeCP2E1 is the major protein isoform in the brain with significantly higher expression in neurons compared to astrocytes [43]. While we reported that MeCP2E1 is highly expressed in seven different brain regions that we studied, MeCP2E2 showed a brain region-specific expression pattern with the highest levels of expression in the cerebellum, highlighting the functional importance of MeCP2 isoforms in the cerebellum [44]. We reported that during differentiation of embryonic brain-derived neural stem cells, *Mecp2*/MeCP2 expression is controlled by DNA methylation, with a reciprocal expression pattern for the two isoforms [45, 46]. Importantly, significant correlations exist between the transcript and protein expression of the

two MeCP2 isoforms with that of DNA methylation at its regulatory DNA sequences (regions 1-6) in adult murine brain [44]. I will further discuss the importance of MeCP2 and its two isoforms in human disease and neurodevelopmental brain disorders in the last section of this book chapter.

DNA Methyl Erasers

For a long time, DNA methylation was considered to be a stable and repressive DNA modification. However, it is now clear that through the action of ten-eleven translocation (TET) proteins (TET1, TET2, and TET3), active DNA demethylation occurs. During this process, TET family members oxidize the methyl group of the 5-mC modification by an oxygen substrate, and this reaction leads to the generation of 5-hmC DNA modification. Such modification (5-hmC) is highly abundant in the brain, in neurons, and in pluripotent embryonic stem cells. Interestingly, the same TET proteins are capable of further oxidizing the 5-hmC mark into 5caC and 5fC (Fig. 1), but currently not much is known about the functional role of these two modifications. Mice deficient for either of *Tet* genes (*Tet1*$^{-/-}$, *Tet2*$^{-/-}$, *Tet3*$^{-/-}$) have normal preimplantation development. While *Tet3*-deficiency leads to neonatal lethality after birth [47], *Tet2*-deficient mice develop spontaneous myeloid leukemia [48, 49], with *Tet1*-deficinet mice only showing a small body size. However, despite being a mild phenotype, this small body size of *Tet1*-deficient mice is noticeable from the post-implantation stage and during embryonic development [50].

Regarding the role of 5-hmC, current research studies suggest that it promotes transcriptional activation due to its high abundance in the intragenic regions of enhancers and transcriptionally active genes in embryonic stem cells [19, 51]. During early development, passive DNA demethylation occurs that is through consequent cellular divisions in the absence of DNA methyl transferases (Fig. 2). During development, there is a global DNA demethylation of 5-mC at the paternal genes, right after fertilization and during the early preimplantation of embryos due to active and passive 5-mC DNA demethylation. The passive 5-mC demethylation coincides with the physical absence of DNMT1 transcription and expression, as well as dilution of DNMT1◦, originating from the oocyte DNMT1 (the protein levels are diluted and reduced while the cells divide). The passive DNA demethylation is due to the DNMT1 absence, and despite the fact that its protein partner, UHRF1 (ubiquitin-like containing PHD and RING finger domains 1), is still available. UHRF1 is the protein partner of DNMT1 that binds to specific DNA sequences, actively recruiting DNMT1 for subsequent enzymatic activity of DNAM1. On the other hand and in parallel, there is an active 5-mC DNA demethylation at the paternal genes that is due to the activity of TET proteins in oxidizing 5-mC to 5-hmC, 5fC, and 5caC. The TET activities during this embryonic time of developmental are mainly the result of TET3 activity, as TET1 and TET2 are not expressed until a later developmental time-point and at the morula stage. After the formation of 5fC and 5caC, a destabilization of the N-glycosidic bond may happen that promotes thymine DNA glycosylase (TDG)

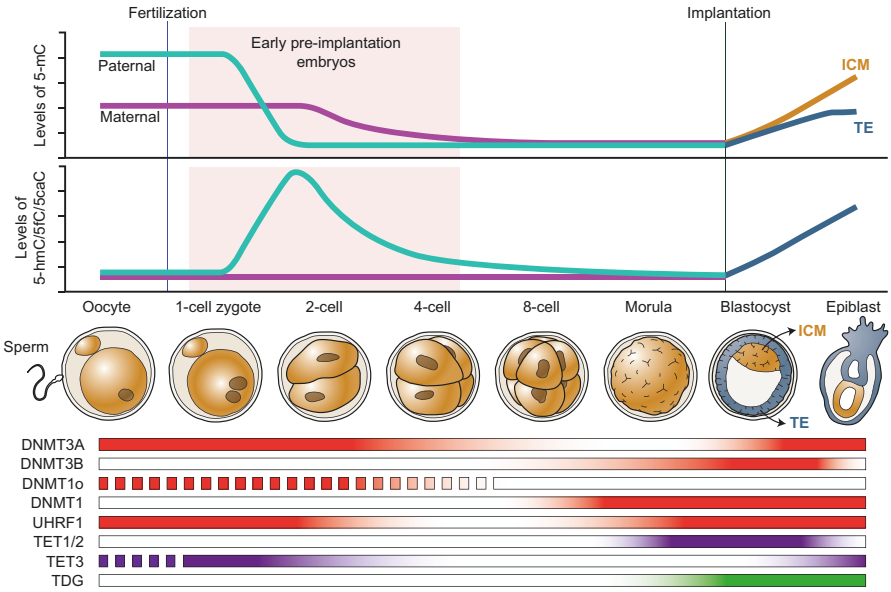

Fig. 2 Global change in different types of DNA methylation in the paternal and maternal genes during the pre-implantation period of development in mouse embryos. The global change in the levels of different types of DNA methylation (5-mC, 5-hmC, 5fC, and 5caC) is shown. The relevant expression patterns of the DNA methyl transferases (DNMT) 3A, DNMT3B, DNMT1, DNMT1o (oocyte-driven DNMT1), UHRF1 (DNMT1 recruiting partner), TET1/2/3, and TDG are shown. Right after the implantation stage, DNA methylation levels are elevated at the inner cell mass (*ICM*) and trophectoderm (*TE*) (Figure is modified and adapted from Wu and Zhang 2014 [52])

activity *via* specific chain of events that lead to base-excision repair (BER) and removal of the modified nucleotide, but such cascade of molecular events is not active after fertilization and until implantation stage due to the TDG absence in the developing embryos. The maternal genes also undergo DNA demethylation with following the paternal 5-mC demethylation, and except for the imprinted genes that maintain their DNA methylation patterns, the rest of the genome within the developing embryonic cells lack DNA methylation till implantation. At this stage of development, 5-mC DNA methylation is restored globally at the inner cell mass (ICM) and trophectoderm (the outer embryonic layer at the blastocyst stage). This is in parallel increase at the levels of 5-hmC, 5fC, and 5caC due to the activity of TET proteins in the embryonic cells and tissues (Fig. 2). For further review of the molecular mechanisms of DNA demethylation during embryonic development, please refer to the following reviews and the references in there [5, 52].

Histone Modifications

Perhaps the most diverse form of epigenetic regulation is being orchestrated through histone post-translational modifications. Histones are highly alkaline DNA-bound proteins that make up the coatomer core of the nucleosomes (two dimers of [H2A-H2B] + 2 molecules of H3 + 2 molecules of H4) enwrapped by two rounds of double-stranded DNA and an additional linker histone H1 that all together constitute the basic structure of the chromatin (Fig. 1). The naked DNA without the basic histone molecules is about 1.8 m in a human somatic cell with 46 chromosomes, but addition of histone molecules would bring it to about 90 μm in the form of chromatin structure within the cellular nuclei [53]. The very first histone PTM that were reported in 1964 consisted of the acetylation and methylation of histone molecules [54]. Today, we know of many other forms of histone PTM beyond the histone acetylation and histone methylation that include histone phosphorylation, isomerization, ubiquitination, ADP-ribosylation, and histone sumoylation (Fig. 1). Such histone modifications are usually at the terminal domain of histone molecules, with an exception of few histone PTM that may also occur within the core part of the histone molecules (i.e., phosphorylation of histone H3 tyrosine 41). Considering the large number of amino acids within each of the core histone tails and the existence of different forms of histone modifications, one can appreciate the complexity and versatility of the information that can be transferred *via* this type of epigenetic marks. To add another layer of complexity, one should also note the existance of mono-, di-, or tri-histone modifications, which may not necessarily mark the same chromatin compartments, and within the di-modifications, there are also potentially symmetric or asymmetric histone PTM that once again may be a signature of different chromatin compartments. Together, with the cross talk of histone PTM and DNA modifications (different forms of 5-mC, 5-hmC, 5fC, 5caC, and CpH methylation), as well as the potential physical masking of one histone PTM by another PTM, and the regulatory role of three-dimensional chromatin structure, chromatin remodeling, and nucleosome positioning, among others, the magnitude and complexity of proper establishment of such orchestrated molecular mechanism in every single cell of the body can be appreciated.

Similar to what was discussed for DNA methylation in the previous sections, there are also "writers," "readers," and "erasers" of histone PTM. Depending on the type of histone PTM, the proteins that are within each group of the "writers," "readers," and "erasers" are different. For example, histone acetylation of lysine amino acids is catalyzed though the activity of K-acetyltransferases (KATs) as "writers." The KAT writers use the cofactor "acetyl-CoA" to transfer the "acetyl" group to the lysine amino acids, thereby catalyzing the formation of histone acetylation. The "reader" molecules such as PCAF and P300 would then bind and interpret this histone PTM, which may subsequently recruit other transcription factors or regulatory molecules to communicate the intended epigenetic signal in transcriptional activation. The histone acetyl "eraser" molecules are grouped as members of histone deacetylase (HDAC) family that belong to HDAC classes I to IV. For a detailed

review about the members of this family of erasers, refer to Liyanage et al. 2015 and the references in there [8]. HDAC inhibitors such as Trichostatin A are commonly used in the treatment of human cancer, by globally inducing histone hyperacetylation. Similar to what I discussed for histone acetylation, there are all three types of epigenetic molecules (writers, readers, and erasers) for other types of histone PTM that are discussed in detail elsewhere [8, 21].

Other Epigenetic Regulatory Mechanisms

Besides DNA methylation, histone modifications, and their cross talk, there are other modes of epigenetic control in place to ensure the proper gene expression program during embryonic development, after birth, and in adult organisms. These include the action of small noncoding RNAs (microRNAs and Piwi-interacting RNAs) in transcriptional silencing and translational regulation, long-noncoding RNAs in transcriptional control, as well as methylation of RNA molecules, nucleosome density at specific genomic loci (promoter regions, transcription start sites, and/or exon-intron boundaries), long-range enhancer-promoter interactions, the functional role of insulators [i.e., CCCTC-binding factor (CTCF) activity], and chromatin remodeling, among others. Such epigenetic mechanisms may have profound and critically important key roles during development. Mis-regulation of these molecular events may lead to impaired cellular function, cancer, and human disease. Perhaps the best-studied example of developmentally important genes that are controlled by such orchestrated cascade of epigenetic mechanisms are the *Hox* gene clusters. The highly conserved *Hox/HOX* gene clusters encode for HOX transcription factors that determine the anterior-posterior and dorsal-ventral patterning of the developing embryos (Fig. 3a). Transcriptional expression of the *Hox/HOX* genes is controlled by combination of DNA methylation marks, histone post-translational modifications, a suggested unidirectional chromatin remodeling (shown experimentally for the *Hoxd4*) [55] (Fig. 3b), enhancer-promoter integrations, nucleosome positioning, and the activity of regulatory RNA molecules. For a detailed review of *Hox/HOX* gene control by epigenetic mechanisms, refer to Barber and Rastegar 2010 and the references in there [12].

Fig. 3 (continued) groups shown below the fly images. Members of each paralogue groups (i.e., *Dfd*, *HOXA4*, *HOXB4*, *HOXC4*, and *HOXD4*) are more similar to each other in comparison of the members from the same HOX cluster (i.e., *HOXA1* to *HOXA13*). Underneath the fetus, a proposed unidirectional chromatin opening at the 3′–5′ unidirectional *HOX* gene activation (colinearity is a specificity of *HOX* genes) is shown. (**B**) A cascade of epigenetic events at the *Hoxd4* locus, *via* sequential histone modifications at the 3′ *Hoxd4* enhancer that reaches to the *Hoxd4* promoter located more 5′ to its enhancer, is shown. Note that the recruitment of CBP (histone acetyl transferase), the transcription factor PAX6, and the initiation of H3K9ac, H3K4me, are followed by H4K4K11K15ac histoneH4 modification from the 3′ end of the gene (enhancer) to the promoter. This is then followed by the recruitment of the transcriptional machinery (RNA polymerase II) through the transcription factors YY1-Mel18 and recruitment of PBX1-HOXD4 to the *Hoxd4 cis*-regulatory elements at the promoter [55–57]. (**A**, **B**) are modified and updated from Barber and Rastegar 2010 [12]

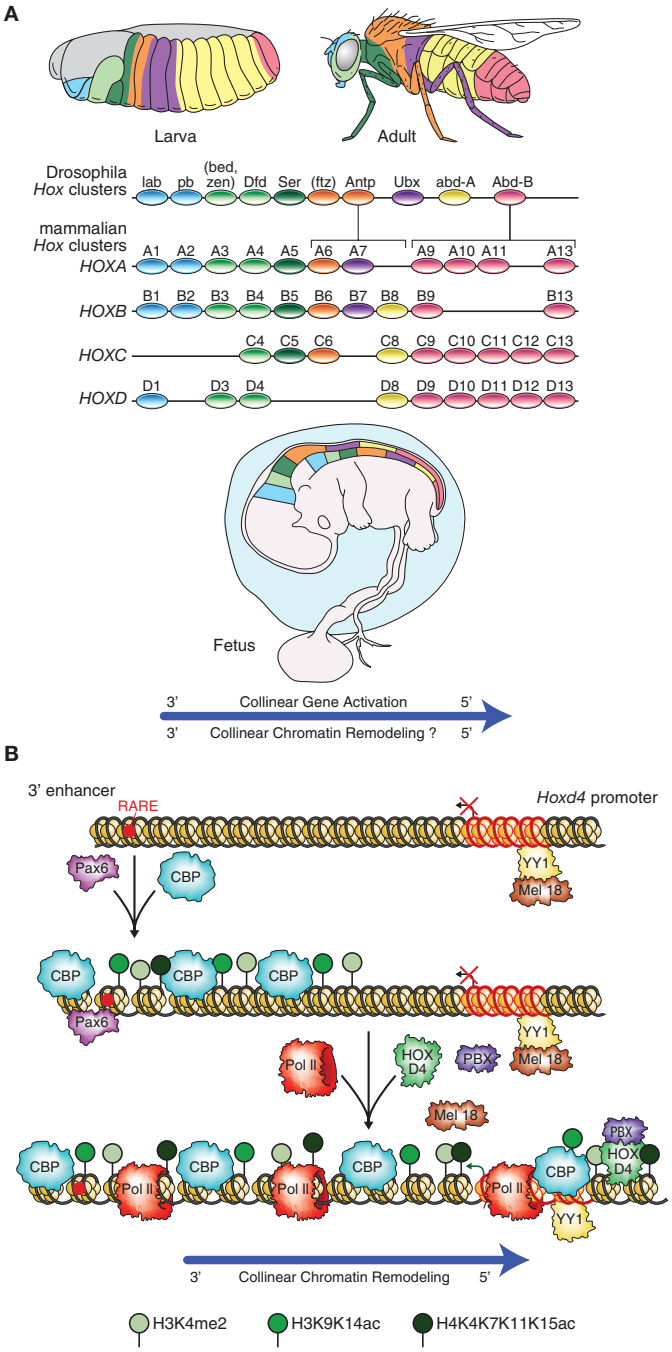

Fig. 3 Schematic illustration of the *Hox/HOX* clusters and unidirectional chromatin remodeling at the *Hoxd4* gene locus. (**A**) The larvae and adult *Drosophila Melanogaster* are shown on the top. The color-coded fly is in harmony with the *Hox/HOX* gene members of the 13 paralogue

Epigenetics in Development and Bivalent Marks

Epigenetics play a profound regulatory role during embryonic development with global changes in DNA methylation, histone modifications, and chromatin structure (discussed already in detail and summarized in Fig. 1). Intense effort from independent research groups has been devoted to understand the biology of global DNA demethylation at both the maternal and paternal genes, which happens as a result of both active and inactive modes of DNA demethylation. As discussed in a previous section on DNA methylation, shortly after zygote formation by fertilization of an egg and sperm (during embryonic preimplantation), paternal genes undergo global DNA demethylation (reduced 5-mC). Such global effect is *via* passive DNA demethylation through cellular division associated with the absence of DNMT1 expression and dilution of oocyte DNMT1 (DNMT1$_{\circ}$) that keeps being less and less during each mitosis. Due to the absence of DNMT1 and reduction of DNMT1$_{\circ}$ during each cell cycle, DNA methylation maintenance is globally reduced. Maternal genes will also undergo similar DNA demethylation (reduced 5-mC), with a short delay following paternal genes. Active DNA demethylation accompanies this process through the catalytic role of TET1/2/3 proteins, leading to an induction of other forms of DNA methylation (5hmC/5fC/5caC) only in the paternal genes. However, maternal genes do not undergo the global active DNA demethylation by TET proteins. Right around implantation, the global DNA methylation will be established through the activity of DNMT proteins in both the inner cell mass (cellular mass in the primordial embryo that will give rise to the embryonic body of the fetus) and trophectoderm (cells of the outer layer of the blastocyst that will generate the extra-embryonic tissues) (Fig. 2). There are global histone modification changes that in collaboration with DNA methylation types and other epigenetic mechanisms tightly control the proper process of embryonic development. Deregulation of these mechanisms caused by genetic mutations or influenced by environmental factors may lead to mild-to-severe consequences in the developing embryo.

To understand early developmental mechanisms that are orchestrated by genetics and epigenetics, researchers have intensely used self-renewing and differentiating pluripotent embryonic stem cells. Through genome-wide chromatin immunoprecipitation (ChIP) studies, scientists have shown that depending on low CpG or high CpG contents of the promoter regions, developmentally important genes (such as *Hox/HOX* genes), pluripotency genes, and housekeeping genes are differentially marked by histone PTM (Fig. 4). It is now over a decade that we know *Hox* genes and some other developmentally important genes are at a poised state of transcription carrying "bivalent marks", within the "bivalent domains" of the genome. In 2006 Bernstein et al. first introduced the concept of bivalent domains and bivalent chromatin structure, referring to chromatin regions that carry histone H3 (lysine) K4 methylation and histone H3 (lysine) K27 methylation, simultaneously [58]. The "bivalent marks" are accordingly referred to the existence of these active (H3K4 methylation) and inactive/repressive (H3K27 methylation) at the N-terminal region of same histone H3 molecule at the regulatory regions of developmentally important

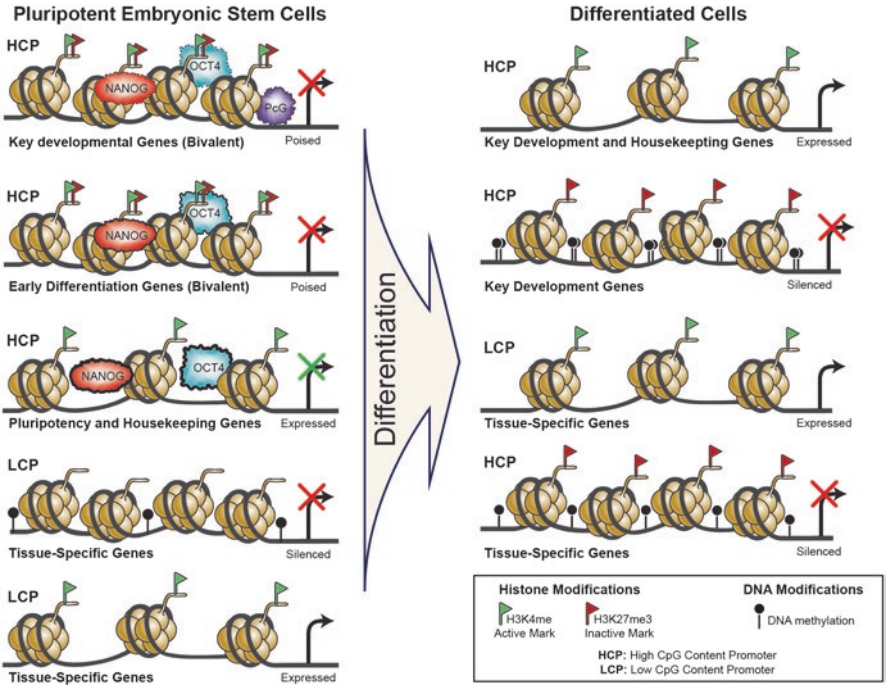

Fig. 4 Presence of bivalent marks in the embryonic stem cells and their resolvation in differentiated cells. Bivalent marks (active:H3K4me3; and inactive: H3K27me3) exist on the N-terminal tail of the same molecule of histone H3 in the pluripotent embryonic stem cells at the high CpG content promoters of the developmentally important genes (e.g. *Hox* genes) and early differentiation genes. The thickness of the black line for NANOG and OCT4 corresponds to the enrichment of these transcription factors/pluripotency factors at the regulatory/promoter gene regions. During embryonic stem cell differentiation, genes that carry bivalent marks either lose the active H3K4me3, keeping the H3K27me3, and may gain DNA methylation marks and become inactive/silenced. On the other hand, genes that should be turned on would become active by losing H3K27me3 histone PTM but would keep H3K4m3 and become active. Note that the overall level of DNA methylation is lower in embryonic stem cells but increases during differentiation. Housekeeping genes remain active in embryonic stem cells as well as in differentiated cells (Figure is modified and updated from Rastegar et al. 2011 [21])

genes [12]. As stated earlier, HOX proteins and their cofactors control the proper patterning of the embryonic central nervous system along the anterior-posterior embryonic axes with key roles in stem cell differentiation [4, 12, 55–57, 59, 60]. As development/embryonic stem cell differentiation proceeds, the bivalent marks at the poised genes are resolved to further gain DNA methylation in combination with keeping the H3 K27 methylation and become silenced, or lose the inactive histone mark, keeping histone H3K4 methylation and relaxed chromatin structure for genes that would become transcriptionally active. In pluripotent embryonic stem cells, the active or silenced state of gene transcription is further regulated by the pluripotency transcription factors; OCT4, NANOG, and members of Polycomb (PcG) transcrip-

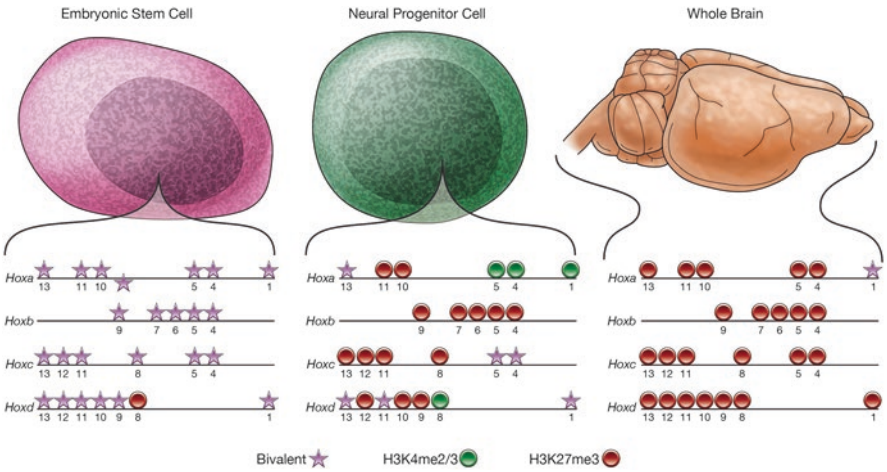

Fig. 5 Bivalent marks at the *Hox* clusters during development of the brain cells. In pluripotent embryonic stem cells, bivalent marks exist at all paralogue members of the *Hox* genes along all four clusters, except for the *Hoxd8*. Later during the differentiation of the brain cells in neural progenitor cells that are generated from the multipotent neural stem cells, most Hox genes lose their bivalent marks, being either active or inactive. In the adult brain, none of the Hox genes carry bivalent marks, except for the *Hoxa1* gene. Stars indicate bivalent marks (the gene locus carries both H3K4me3 and H3K27me3); *red circles* indicate inactive genes (carrying H3K27me3); and *green circles* show active genes (carrying H3K4me3). Figure is modified and updated from Barber and Rastegar 2010 [12]

tional silencing proteins. The pluripotency genes are further controlling their own expression through a regulatory feedback loop during embryonic stem cell self-renewal. For a detailed review on the bivalent marks please refer to the following reviews/book chapters and the references in there [5, 21]. Depending on the process of differentiation towards different cell fate commitments, the epigenetic marks and transcription factor binding at the *cis*-regulatory elements would change in order to ensure proper gene expression program of the individual cells. Once the central nervous system and the brain development process is completed, the bivalent marks barely exist at the *Hox/HOX* genes, such as the only remaining *Hox* gene with a bivalent modification is *Hoxa1* (Fig. 5).

Epigenetics and Cerebellum

Epigenetic mechanisms control the proper development of all brain regions including the cerebellum. The word "cerebellum" is rooted from a Latin word that means "little brain." In general, the mammalian cerebellum is a distinct structure that is situated underneath of the brain in the posterior cranial fossa (see chapter "The Embryology and Anatomy of the Cerebellum"). Anatomically the cerebellum has a

distinguishable structure of neurons within its granular layer, the molecular layer, and a layer of the Purkinje cells. The proper function of cerebellum is critical and essential for motor coordination of the body. Cerebellum also plays important roles in cognitive function including attention and language while controlling emotional responses such as reaction to pleasure and fear. The process of murine and human cerebellum development is controlled by a combination of epigenetic mechanisms and particular gene regulatory networks [61]. Among different modes of epigenetic mechanisms, DNA methylation has attracted much attention in cerebellum development and function. Within the different cell types of the cerebellum, 5-hmC is markedly enriched at the euchromatic genomic regions, and 5-mC is found at the heterochromatin compartments with MeCP2 being the main reader of both 5-hmC and 5-mC in the brain [39]. In the adult mouse brain, reports from my own lab indicate that both MeCP2E1 and MeCP2E2 isoforms are expressed in neurons, astrocytes, and oligodendrocytes. However, expression of the minor MeCP2 isoform (MeCP2E2) reaches to its highest level in the cerebellum with almost comparable levels to the major MeCP2E1 isoform [44].

Epigenetics and Neurodevelopmental Cerebellar Disorders

Deregulation of epigenetic mechanisms in the brain and cerebellum is associated with neurodevelopmental and cerebellar disorders. Research by independent groups has established the involvement of epigenetic mechanisms in fetal alcohol spectrum disorders (FASD) (see chapter "Teratogenic Influences on Cerebellar Development"), autism spectrum disorders (ASD) (see chapter "Neurodevelopmental Disorders of the Cerebellum: Autism Spectrum Disorder"), fragile X syndrome, and Rett syndrome (RTT). Of different types of epigenetic mechanisms that I have discussed here, altered DNA methylation and more specifically cerebellar change in 5-hmC levels are linked to Fragile X Syndrome. Both ASD and FASD as cerebellar neurological disorders are discussed in detail in other chapters of this book.

In general, cerebral dysfunction is an established trademark of FASD and it appears that the second and third trimester-equivalent ethanol exposure in mice highly influence Purkinje cells of the developing cerebellar vermis relative to interneurons. Besides the reduced number of GABAergic/glycinergic neurons due to ethanol exposure, decreased lobule volumes in cerebellar vermis of the developing mice is also evident subsequent to in utero ethanol exposure in the developing mouse embryos. The reduced volume of most cerebral lobules is also found as an outcome of significant decrease in the number of cells, dendritic arborizations, and/or axonal projections [62]. DNA methylation is reported to play a profound role in FASD pathobiology, detected in FASD patients, and during stem cell modeling of this disease by others and us [45, 63–65]. The associated genetics and epigenetics of FASD are discussed in detail elsewhere [66], and impaired cerebral function in FASD is also covered in detail in another chapter of this book.

Studies in human post-mortem brain indicate that increased MeCP2 recruitment at the glutamic acid decarboxylase 67 (*GAD1)* and Reelin (*RELN)* gene promoters happens *via* enrichment of 5-hmC DNA methylation and a mirror decrease of 5-mC in the cerebellum of ASD patients [67]. Such epigenetic change and altered MeCP2 binding was not detected at the *GAD1* and *RELN* gene bodies of ASD patients in the same examined cerebellum samples. While the protein product of *GAD1* gene is involved in gamma-aminobutyric acid (GABA) synthesis, REELIN is dominantly expressed in GABAergic neurons. In post-mortem brain tissues of ASD patients, GABA neurotransmitters are reduced levels in the cerebellum [68, 69]. Research by independent groups has suggested the role of DNA methylation in autism spectrum disorders and potential application of DNA methyl inhibitors for ASD [46, 70, 71].

In a recent study in human cases of suicides compared to control individuals, reduced connexins (*CX*); *CX30* and *CX43* levels were found to be associated with increased histone H3K9 methylation in the prefrontal cortex, but such observation was not consistent and were not detected in the cerebellum. The authors concluded that extensive cerebral astrocyte dysfunction is associated with major depressive disorders [72]. Recent studies further introduce DNA methylation as an emerging marker for cerebellar epigenetic age [73]. Such research highlights the importance of DNA methyl-related proteins, which once again brings MeCP2 to the forefront of research in this field and further link MeCP2 as the major DNA methyl-binding protein in the brain to age-related cerebellar disorders. Ataxia-telangiectasia is a genetically inherited neurodegenerative disorder that is caused by *ATM* loss-of-function mutations. This gene encodes for a protein kinase that has key roles in DNA damage response for DNA double-strand breaks. One of our today's challenges in understanding Ataxia-telangiectasia is based on our limited knowledge on the functional roles of Purkinje cells in this regard, and specifically on their vulnerability to *ATM*-deficiency. However, recent studies have shown significantly reduced levels of 5-hmC in the cerebellar Purkinje cells of $Atm^{-/-}$ mouse cerebellum and human Ataxia-telangiectasia cerebellum tissues directly related to compromised TET1 enzymatic activity. It is further suggested that loss of 5-hmC is critically important in mediating the susceptibility of Purkinje cells to *ATM*-deficiency [74].

Within the neurodevelopmental and cerebral disorders with an epigenetic link, perhaps one of the most-studied diseases would be Rett Syndrome (RTT). RTT is a severe neurological disorder that in more than 95% of cases is caused by de novo mutations in the X-linked *MECP2* gene [75]. In addition to RTT, *MECP2* mutations are also associated with a broad spectrum of neurological disorders, including X-linked mental retardation, Angelman's Syndrome, severe neonatal encephalopathy, and autism [76–79]. Aberrant MeCP2 expression in the brain also leads to compromised brain function and autism [80]. Currently, Rett Syndrome has no effective treatment; but reactivation of the *Mecp2* gene after the onset of the phenotypes in RTT mouse models partially rescues physiological and anatomical abnormalities [81, 82]. MeCP2 function is dose-dependent, and its loss-of-function or gain-of-function mutations cause overlapping neurological phenotypes and autistic features. In transgenic mice, deficiency in the major MeCP2 isoform (E1-deficiency) is sufficient to mimic RTT-associated phenotypes [83]. To date, there have been many

attempts in finding therapeutic strategies for Rett Syndrome by independent research groups. Perhaps one of the first reports in this regard was the development and validation of regulated gene therapy vectors for both *MECP2E1* and *MECP2E2*. These gene therapy vectors were tested for proper gene delivery into primary neurons, as well as self-renewing adult and embryonic neural stem cells, their differentiated progenies into neurons and astrocytes, and ex vivo delivery into the brain microenvironment of a RTT mouse model [84]. These studies have established that despite MeCP2 functional role in epigenetic silencing of gene therapy vectors in stem cells [85], an efficient and long-term delivery of MeCP2 by this approach is possible in brain-derived neural stem cells and their differentiated progenies into neurons and astrocytes [84]. This was perhaps the first study to support the rescue role of MeCP2E1 in correcting the impaired morphology of *Mecp2*-deficint neurons [84]. Subsequent studies from independent groups indicated that both MeCP2E1 and MeCP2E2 are capable of rescuing the RTT-associated phenotypes in mice but with different efficiencies [86]. While MeCP2 is the main DNA-binding protein in the brain, studies by my team have also shown that its own expression in the developing and adult murine brain is greatly influenced by DNA methylation, involving both 5-mC and 5-hmC [44–46]. Currently, intense research from us and other scientists is focused on the regulation of MeCP2 isoforms and the redundant and nonredundant functional role of the two isoforms in RTT and other neurodevelopmental cerebral disorders.

Conclusions

Epigenetics control and dictate the identity of individual cells during each cellular division. During development, the cellular programing of the developing fetus is orchestrated through epigenetic modifications that are mainly embedded within the chromatin structure and are vulnerable to environmental factors, such as in utero alcohol exposure. Most epigenetic mechanisms are reversible and can be targeted by chemical compounds and drugs, which are attractable routes for therapy strategies. The brain is a very complex organ of the body with billions of functional nerve cells as well as other supportive cell types. The process of cerebellum development is tightly controlled during development, and deregulation of the involved regulatory mechanisms causes human disease. In this regard, the involvement of epigenetic mechanisms and mainly DNA methylation is becoming the center of focus in today's research.

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Hormonal Regulation of Cerebellar Development and Its Disorders

Noriyuki Koibuchi

Abstract Cerebellar development and plasticity involves in various epigenetic processes that activate specific genes at different time points. Such epigenetic influences include hormonal signals from endocrine cells. Various hormone receptors are expressed in the cerebellum, and cerebellar function is greatly influenced by hormonal status. The aim of this chapter is to introduce several key features of hormones and their receptors involved in the regulation of cerebellar development and plasticity. Furthermore, cerebellar developmental disorders caused by aberrant hormonal status are also discussed. This chapter also covers the effect of endocrine-disrupting chemicals that may alter hormone functions in the cerebellum.

Keywords Steroid hormone • Thyroid hormone • Nuclear receptor • Critical period • Endocrine-disrupting chemicals

Hormone and Cerebellar Development: A General Overview

To understand the functional organization of the central nervous system (CNS), including the cerebellum, it is important to consider the process by which neurons differentiate to establish their role and interact with specific target cells to form functional pathways. The development of the brain involves epigenetic processes that activate specific genes during different time frames. As shown in Fig. 1, epigenetic influences that regulate brain development may originate from the neuronal cell itself or from outside of the CNS. The former includes spatial and temporal pattern of intrinsic gene expression tightly regulated by their molecular programs. The latter includes sensory inputs, mediated by the peripheral nervous system and hormonal influence from endocrine cells. These are also crucial stimuli for brain development. Environmental influences, such as stressors, endocrine-disrupting chemicals (EDCs), and undernutrition may affect such processes.

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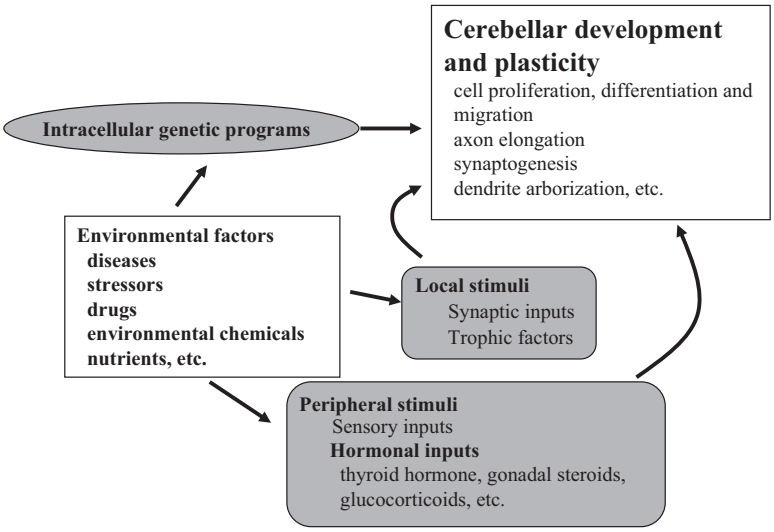

Fig. 1 Schematic diagram showing genetic and epigenetic influences and its modulation by environmental factors involved in cerebellar development and plasticity

The cerebellar cortex forms well-organized structures involving a highly specific and uniform arrangement of cells and microcircuitry [1]. The cerebellum is one of the few sites in the CNS where the pattern of intrinsic connections is known in considerable detail. These features make the cerebellum an ideal system to study the mechanisms of neural development and plasticity. Based on such advantages, many excellent works have been done at various levels ranging from basic science to clinical disorders. In contrast, although a number of hormone receptors are expressed in the cerebellum, and cerebellar function is greatly influenced by hormonal status, a relatively smaller number of studies have evaluated the role of hormonal signaling on development and plasticity of the cerebellum.

Among circulating hormones, a group of small lipophilic hormones such as steroids (corticosteroids, progesterone, androgens, and estrogens) and thyroid hormone (TH) may particularly play an important role in mediating environmental influences. Because of their chemical nature, these are able to cross the blood-brain barrier (BBB) more easily than peptide hormones, although the existence of specific transporters has been proposed [2]. Receptors for such lipophilic hormones are mainly located in the cell nucleus (nuclear receptor, NR) and represent the largest family of ligand-regulated transcription factors [3]. As shown in Fig. 2, the molecular structure of the NR superfamily is homologous. It consists of a highly variable N-terminal domain, which contains a transactivation domain (activation function-1, AF-1), DNA-binding domain (DBD), and ligand-binding domain (LBD). The DBD is the most homologous among these domains. The LBD, which also shares certain homology among NRs, is also responsible for dimerization of NRs and ligand-dependent transactivation (activation function-2, AF-2). To activate or repress the

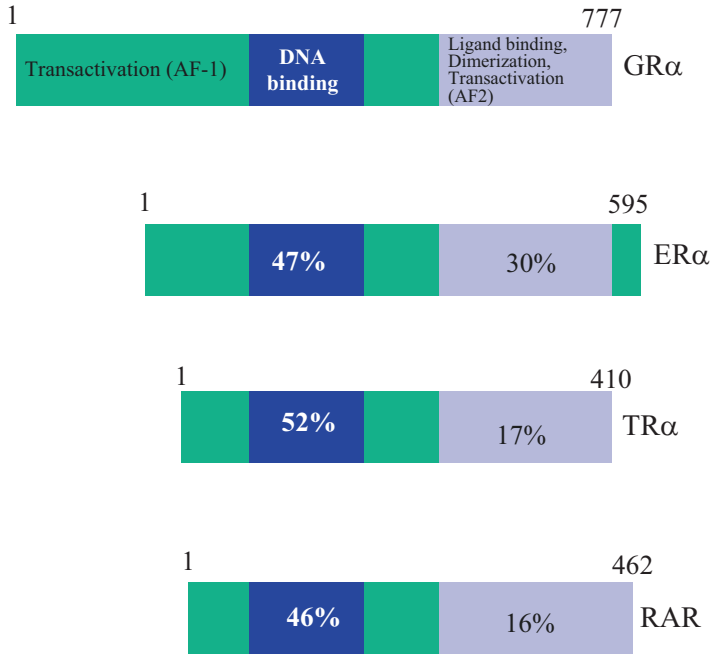

Fig. 2 Protein sequence homology among representative nuclear hormone receptors. *ERα* estrogen receptor alpha, *GRα* glucocorticoid receptor alpha, *RAR* retinoic acid receptor, *TRα* thyroid hormone receptor alpha. Numbers indicate amino acid number

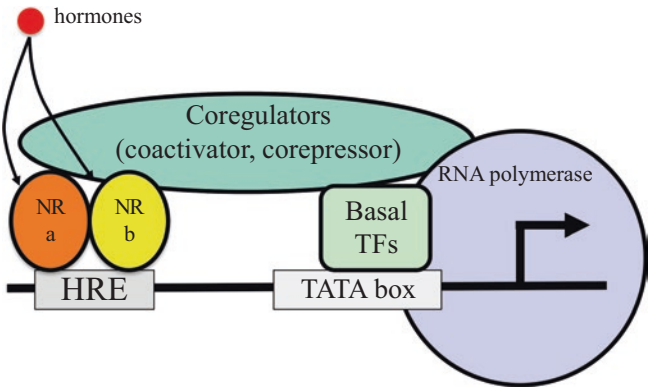

Fig. 3 Interactions of nuclear receptor (*NR*), transcriptional coregulators, and basal transcriptional machinery such as basal transcription factors (*TFs*). NRs bind to hormone response element (*HRE*) located in the promoter region of target genes

transcription of target gene, NRs bind to a specific nucleotide sequence called the hormone response element (HRE) located in the promoter region of target genes (Fig.3). Then NRs recruit a variety of coregulators in a ligand-dependent manner, such as coactivator and corepressor complexes, which then modulate chromatin

structures [4]. With a specific pattern of expression, the NRs are widely distributed in the CNS, as well as in other organs [5]. In the cerebellum, NRs are expressed in a specific temporal and spatial pattern [6]. However, the role of these NRs on cerebellar development and function is not fully understood.

Among the lipophilic hormones, involvement of TH (triiodothyronine [T_3] and thyroxine [T_4]) on cerebellar development has been well studied. Deficiency of TH during the postnatal development results in abnormal cerebellar morphogenesis in rodents [7–9] and humans [10]. Conversely, although the importance of gonadal steroids such as estrogen, progesterone, and testosterone on the development and functional maintenance of the CNS has been well documented, the cerebellum is considered to be relatively insensitive to gonadal steroids. However, recent studies have clarified that gonadal steroids play an important role in cerebellar development and may be involved in various health and disease states [11]. In addition to the supply from circulation, these gonadal steroids are produced locally within the Purkinje cells [12]. Corticosteroids, particularly glucocorticoid, are crucial for the maturation of various organ systems, including the brain [13]. Furthermore, since recent studies have shown the critical role of the cerebellum on social, cognitive, and emotional behaviors [14]; other studies on the role of glucocorticoids on cerebellar development are currently underway. Additionally, it should be noted that these thyroid/steroid hormone-mediated pathways can be disrupted by prescription drugs and environmental chemicals [15].

This chapter will provide useful information regarding the hormonal regulation of cerebellar development and plasticity. Furthermore, cerebellar developmental disorders caused by aberrant hormonal status are also discussed.

Cerebellar Disorders Induced by Aberrant TH Systems

The importance of T_3 and T_4 in brain development has been well documented [7–9]. Deficiency of THs during fetal and early postnatal period results in severe mental retardation. In humans, this is known as cretinism [10]. In the 1980s when newborn screening was introduced in many countries, the initial prevalence of cretinism was 1/3,000–1/4,000 births worldwide; however, recent studies have shown that the prevalence has increased to 1/1,400–1/2,800. This increase may be attributed to the change in diagnostic strategy from serum T_4 measurement to thyrotropin (TSH) measurement, allowing the identification of milder cases. If the diagnosis of cretinism is delayed, the risk of mental retardation and neurologic sequelae, such as poor motor coordination, ataxia, spastic diplegia, muscular hypotonia, strabismus, learning disability, and diminished attention span, is likely to increase.

T_4 enters the brain through the BBB more easily than T_3, an active form of TH [16]. After crossing the BBB, T_4 is taken up by astrocytes and deiodinated to produce T_3 by type 2 iodothyronine deiodinase [17]. T_3 is then transferred to neurons or oligodendrocytes, possibly via monocarboxylate transporter 8 (MCT8) [18]. The effects of THs are mainly exerted through the nuclear TH receptor (TR). At least three TR isoforms are expressed in the CNS (TRα1, TRβ1, and TRβ2) [19].

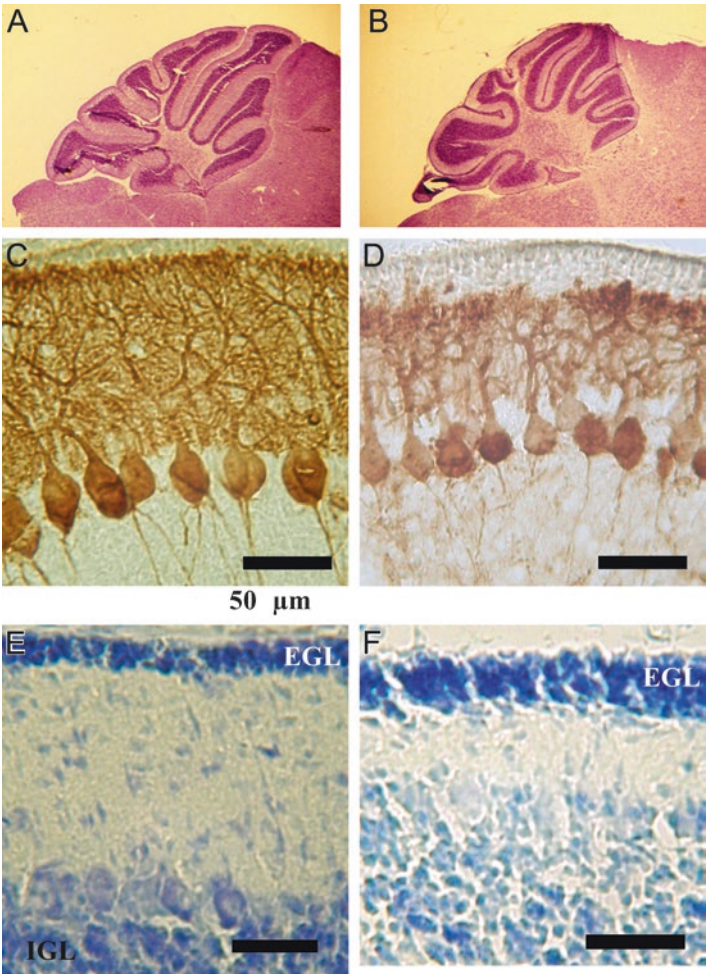

Fig. 4 Effect of congenital hypothyroidism in rat model. Rdw congenital hypothyroid rat, which harbors mutated thyroglobulin gene, shows delayed cerebellar development (**b**, **d**, **f**) compared to control animal (**a**, **c**, **e**). Note the decrease in dendrite arborization of Purkinje cell (**d**) and delayed disappearance of the external granule cell layer (EGL) (**f**)

Perinatal hypothyroidism dramatically affects cerebellar morphogenesis and function. In an animal model of perinatal hypothyroidism, the growth, dendritic arborization, and dendritic spines of Purkinje cells are all markedly decreased. Synaptogenesis between Purkinje cells and parallel fibers is dramatically repressed. The disappearance of the external granule cell layer is postponed as a result of the delayed proliferation and migration of the granule cells into the internal granule cell layer (Fig. 4) [7–9]. TRs are expressed in most subsets of cells in the developing cerebellum in both rodents and humans [20, 21]. TRα1 is abundant in granule cells, whereas TRβ1 is mainly expressed in Purkinje cells. In perinatal hypothyroidism,

the expression of many cerebellar genes is altered [8]. Representative TH-responsive genes in the cerebellum include neurotrophins such as nerve growth factor, BDNF, NT3, and NT4/5, and receptors such as the inositol triphosphate 3 receptors, and retinoic acid receptor-related orphan receptor α, hairless, and myelin basic protein genes. The THs regulate the expression of many of these genes only during a limited period of development. Various animal models harboring TR mutation have been used to study the role of TR in cerebellar development [22]. Interestingly, TRα knockout mice, TRβ knockout mice, and TRα/TRβ double knockout mice do not display obvious cerebellar defects, suggesting that most of the consequences of congenital hypothyroidism in the brain are caused by the detrimental activity of unliganded TR. In fact, in animal models expressing dominant-negative TR, which cannot bind to TH, cerebellar phenotypes, such as disrupted motor coordination, are evident [23–26], suggesting that unliganded TR may cause aberrant phenotypes. In human cases of resistance to TH (RTH) caused by mutation of TR genes, the clinical phenotype is highly variable [27, 28]. This probably depends on the severity of the mutation. However, abnormal motor coordination, which is always evident in animal models, is not common in human cases. Their representative neurological symptoms are emotional disturbances and hyperkinetic behavior [27]. Although the involvement of the cerebellum on such behavioral alterations is also known as cerebellar cognitive affective syndrome [29], further study is required to clarify such phenotypic differences among species.

In addition to cretinism and RTH, recent studies have shown another congenital disease induced by aberrant TH system. Another human disorder related to the TH system is Allan-Herndon-Dudley syndrome, which is an X chromosome-linked disease. The symptoms are hypotonia, dysarthria, athetoid, or other distal limb movements, muscle hypoplasia, and severe mental retardation [30]. Linkage studies have identified the gene locus in Xq 13.2. This region encodes for MCT8 that transports T_3 into the neurons [31]. Animal studies have shown the disruption of cerebellar development by knocking down MCT8 in the Purkinje cells [32]. Although MCT8 is responsible for the TH transport into neurons, the phenotype of Allan-Herndon-Dudley syndrome is much more severe than that in a patient with cretinism or RTH. Thus, further study is necessary to clarify whether this syndrome is induced only by disrupted TH transport or by other additional factors.

Cerebellar Disorders and Gonadal Steroids

Although the importance of gonadal steroids, such as estrogen, progesterone, and testosterone in the development and functional maintenance of the brain, has been well documented, the cerebellum has been previously considered relatively insensitive to gonadal steroids. However, recent studies have clarified that gonadal steroids play an important role in cerebellar development and may be involved in various health and disease states [11]. Aside from the supply from circulation, these gonadal steroids are also produced locally within the Purkinje cells [12].

Testosterone and estradiol (E2) are the two major gonadal steroids synthesized in the testes and the ovaries, respectively. During brain development, gonadal steroids regulate the formation of structures of many brain regions. In late embryonic period, the testes in males start producing testosterone. Because of their lipophilic nature, steroids can pass across the BBB by simple diffusion [33]. Testosterone is then converted to E2 by an aromatase. In contrast, ovaries in females differentiate much later during development and do not secrete E2 during this period. Thus, during the perinatal critical period, there are significantly higher levels of E2 in males compared to females. These are thought to act on male brain development [34]. E2 regulates apoptosis to produce sexually dimorphic cell numbers, dendritic spine formation, neuronal migration, and synaptic organization in the hypothalamic regions, most of which are key regions for regulating male- and female-sexual functions in the adult brain. Because of the lack of estrogen exposure during the perinatal period, the female brain is thought to develop without involvement of E2. However, studies of aromatase gene using knockout mice have suggested that E2 produced by the ovaries during a prepubertal period plays a role in the differentiation of the female-typical brain [35].

In addition to estrogen, androgens, particularly testosterone, directly acting on the androgen receptor (AR), are also thought to play a role in brain masculinization. This is based on studies of human patients with complete androgen insensitivity syndrome and on patients with mutations in the aromatase gene, as well as on studies of rodents with the testicular feminization mutation, which produces a nonfunctional AR [36].

Gonadal steroids also play an important role in the development of the cerebellum. Two nuclear estrogen receptors (ERα and ERβ) were detected in an immature cerebellar granule cell line derived from late embryonic mouse cerebellum [37]. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) studies have shown that both receptors are expressed in the cerebellum from birth to adulthood but levels of ERβ mRNA are significantly higher than those of ERα in neonatal rats [38]. Nevertheless, ERα levels are higher than those in adults during the neonatal period [38]. ERα is predominantly expressed in the Purkinje cells [39]. In contrast, the level of ERβ protein decreased transiently at P5 and P7 in rodents and then increased dramatically at P10 followed by a subsequent decrease to adult levels [40]. ERβ immunoreactivity was detected in various neurons, including Golgi, Purkinje, and basket cells, and the expression in each cell type occurs on different postnatal days. Additionally, differentiating external granular layer cells and glial cells also show ERβ immunoreactivity. Differential expression profiles of ERα and ERβ suggest that E2 exerts its actions in a cell type-specific manner via binding to the two ERs, which play distinctive roles in cerebellar development. Additionally, there may be a possibility that estrogen acts rapidly through a membrane-associated receptor in the developing cerebellum [41].

As discussed above, during the late embryonic period, E2 converted from testosterone may be major gonadal steroid that may have some effect in the developing cerebellum. Previous studies showing the expression of aromatase in mid gestation in monkey [42] and early postnatal age in rat [43] support this hypothesis. Then at

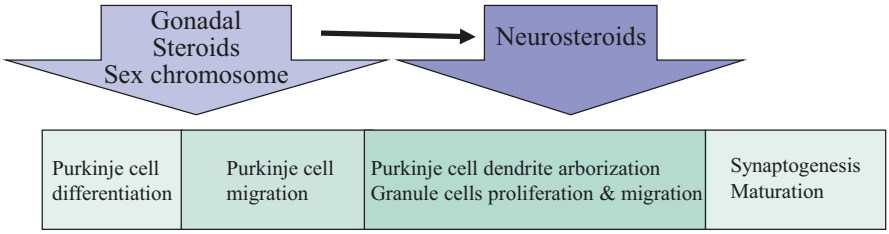

Fig. 5 Possible differential roles in gonadal steroids and neurosteroids during cerebellar development

the later stage, the estrogen level in the cerebellum increases relative to that in the plasma [44] with the expression of enzymes responsible for estrogen [43] and progesterone [45] synthesis, indicating that gonadal steroids are locally produced as "neurosteroids." The most evident action of gonadal steroid is that estrogen and progesterone promote dendritogenesis and increases dendritic spine density [44, 46]. Taken together, gonadal steroids produced in the testes or ovaries may play an important role during the early cerebellar development. Then, de novo synthesized neurosteroids may play a major role at a later stage of development. Additionally, possible sex chromosome effects have been proposed [47]. The diagram showing the influence of gonadal steroids on cerebellar development is shown in Fig. 5.

Whether there are any sex differences in cerebellar architecture remains controversial. Some magnetic resonance imaging (MRI) studies have reported that the cerebellar size in men, both adults [48] and children [49], is larger than that in women, and other MRI studies failed to detect such differences [50]. Biochemically, the levels of aromatase and several enzymes related to estrogen synthesis are higher in postnatal male rats than in females [43], whereas calbindin levels are higher in female mice [47]. While these are only few examples related to sexual differences in the cerebellum, sexual dimorphism is not evident in gene expression patterns in the cerebellum.

In spite of the fact that no clear sex differences in cerebellar morphology and gene expression were observed, there is a clear sex difference in cerebellar pathology in several developmental diseases in humans and related animal models. For example, the prevalence of autism is four times higher in men [51], and autistic patients commonly show increased cerebellar volumes during childhood and hypoplasia in adult [52, 53]. In postmortem tissue in autistic patients, Purkinje and granule cells were reported to be lower in number [54, 55]. Another clinical example is attention-deficit hyperactivity disorder (ADHD), which affects two to four times more males than females [56]. Untreated children show the decreased volume of the posterior inferior vermis [57]. In our animal model, when polychlorinated biphenyl (PCB), an environmental chemical pollutant and developmental neurotoxicant, is administered postnatally to dams, pups present ADHD phenotype [58]. Hyperactivity was more evident in males. Additionally, motor coordination was more severely disturbed in male rats (Fig. 6) [58]. More recently, the change in the volume of several cerebellar regions in transgender individuals has been reported, although the mechanisms underlying such cerebellar structural difference are unknown [59, 60]. To clarify the molecular mechanisms of sexual differences in cerebellar pathology, further study is necessary.

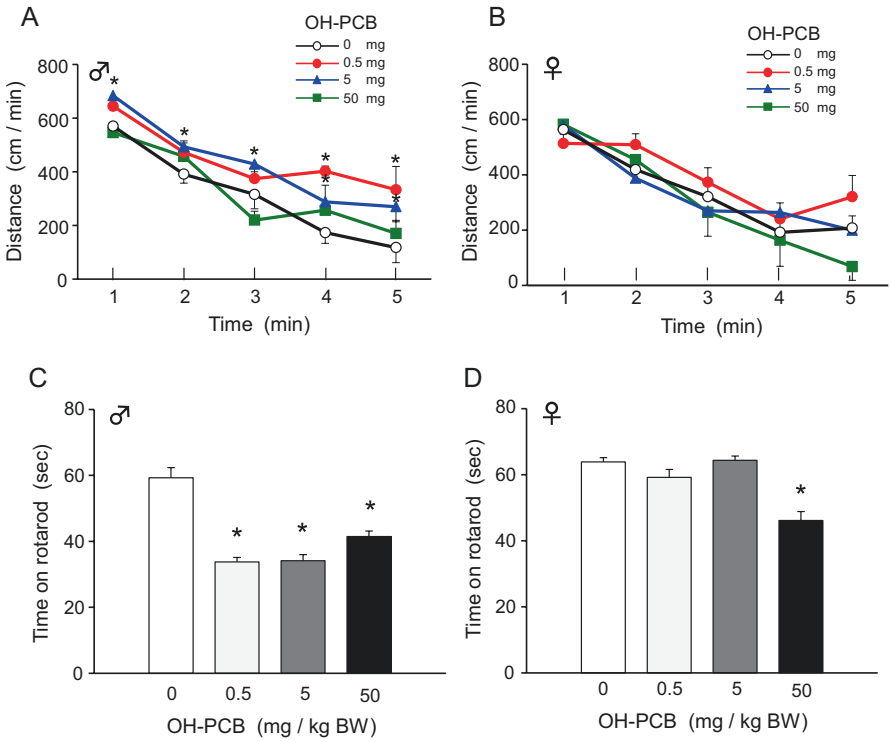

Fig. 6 Sexual difference in the effect of perinatal exposure to hydroxylated polychlorinated biphenyl (OH-PCB106). PCB was orally administered to the dam every other day from postpartum day 3–13 [58]. (**a**, **b**) Effects of PCB on locomotor activity in the open field in male (**a**) and female (**b**) rat. (**c**,**d**) Effect of PCB on motor coordination on rotarod in male (**c**) and female (**d**) rat. Note that behavioral alteration was more evident in male. $P < 0.05$ vs control (no PCB)

Cerebellar Disorders Induced by Corticosteroids

Glucocorticoids and mineralocorticoids are major adrenal steroid hormones (corticosteroids) synthesized in the adrenal cortex. Mineralocorticoids regulate sodium and potassium levels, whereas glucocorticoids are involved in the stress response and carbohydrate metabolism. Glucocorticoid levels are controlled through the hypothalamic-pituitary-adrenal (HPA) axis, whereas mineralocorticoid levels are regulated by the renin-angiotensin-aldosterone system. The effect of corticosteroids in the brain is mainly exerted through binding to intracellular receptors, the glucocorticoid receptor (GR), and mineralocorticoid receptor (MR) [61]. Although GR binds preferentially to glucocorticoids, MR can bind to both glucocorticoids and mineralocorticoids with similar affinity. The specificity of MR is determined by the colocalized expression of 11 β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which inactivates cortisol [61]. Additionally, rapid effects that respond within minutes are regulated by non-genomic action [62].

In most mammalian species, the glucocorticoid concentration increases dramatically during the perinatal period, and such increases are associated with the maturation of several organs including the lungs and brain [63]. In the developing CNS, corticosteroids regulate neurogenesis, neuronal morphology, and function in response to chronic stress. During fetal rat brain development, GRs are expressed widely, including cerebellum, with high levels of 11β-HSD2 and much lesser levels of MR [64], indicating that the developing cerebellum is protected from excess glucocorticoids. In the early postnatal rat cerebellum, however, the MR expression in Purkinje cell become evident, followed by the GR expression within this cell type and MR expression in migrating granule cells, the internal granule layer, and the deep cerebellar nuclei [65]. Conversely, 11β-HSD was specifically expressed in the external granule cell layer [66], indicating that MR as well as GR may mediate postnatal glucocorticoid action in the cerebellum. Prenatal glucocorticoids influence the development of Purkinje neurons [67]. Furthermore, the glucocorticoid-binding capacity of the neonatal rat cerebellum (P8-P15) is highest among brain regions, such as the cerebral cortex, hippocampus, and olfactory bulb [68]. These results indicate that glucocorticoids play an important role in the developing cerebellum to induce multiple changes in response to various environmental stimulations.

As discussed above, studies of rodents have shown that the cerebellum has higher glucocorticoid binding capacity on P8-P15 [68], which is equivalent to the human perinatal period. Such a high sensitivity to glucocorticoid stimulation may make the cerebellum susceptible to development alterations if glucocorticoid homeostasis is disrupted by perinatal stress or glucocorticoid administration. In rats, cortisone treatment during the prenatal [69] and postnatal [70] development resulted in a decreased number of cerebellar granule cells. Such a decrease may be caused by an increased sensitivity to oxidative stress by perinatal glucocorticoid treatment, inducing cell death [71]. In humans, premature newborns suffering from respiratory distress caused by lung immaturity or mothers at a risk of premature delivery before 34 weeks of gestation are sometimes administered glucocorticoid therapy. Newborns who received such treatment sometimes show neuromotor/cognitive disorders [72], including abnormal cerebellar development [73]. Thus, careful use of glucocorticoid therapy (i.e., dose and timing) is required for fetuses and newborns.

Stressful experiences in the prenatal or early postnatal period may increase the risk of neurological and psychiatric disorders, such as ADHD, autism, schizophrenia, and depression [74]. The cerebellum is one of the major brain regions to be directly affected by stressful experience, and the involvement of glucocorticoid system has been proposed as the culprit for such abnormalities [75]. Maternal deprivation (MD) during the early postnatal period in rats causes retardation in the development of cerebellar-dependent motor coordination and behavioral abnormalities similar to those in schizophrenia [76]. In MD rat, a transient increase has been reported in several neurotrophic factors, such as brain-derived neurotrophic factor, TrkB, and oligodendrocyte-myelin glycoprotein [77]. These results support the possibility that abnormally increased levels of glucocorticoids caused by neonatal stress during development are associated with structural abnormalities in the cerebellum, leading to psychosomatic abnormalities in adulthood. However, in spite of high glucocorti-

coid binding capacity in the developing cerebellum, the role of glucocorticoid during cerebellar development has not yet been fully clarified. Further investigations, including studies with human subjects, are necessary.

Environmental Chemicals That May Disrupt Cerebellar Development Through Disruption of Hormone Actions

As discussed above, various hormones are involved in cerebellar development and disruption of such hormonal environment may affect such development. A large number of synthetic or natural chemicals may disrupt hormonal environment. These are referred to as EDCs. The exact definition of an EDC by the World Health Organization (WHO) is as follows: "An endocrine disrupting chemical is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or (sub) populations" [78]. As many hormones have distinct effects, specifically in critical periods during development, fetal or early neonatal exposure to such chemicals may induce adverse effect in various organs, including the CNS [79]. Recent advances in EDC research have provided many important data regarding the neurotoxicity of such EDCs [80]. Table 1 shows representative EDCs that are categorized as pharmaceuticals, herbicides, fungicides, insecticides, industrial chemicals and byproducts, and organic and inorganic metals [79, 80]. Importantly, although there are approximately 1,000 EDCs, more than 100,000 chemicals exist in the environment. The

Table 1 Environmental chemicals showing hormonal or antihormonal activities

Classification	Chemicals
Pharmaceuticals	Hormones or antihormones, amiodarone, DES, fenamate, phenobarbital, phenytoin
Herbicides	2,4,-D, 2,4,5,-T, alachlor, amitrole, atrazine, linuron, metribuzin, nitrofen, trifluralin
Fungicides	Benomyl, ethylene thiourea, fenarimol, hexachlorobenzene, mancozeb, maneb, metiram – complex, tri-butyl-tin, vinclozolin, zineb
Insecticides	Aldicarb, beta-HCH, carbaryl, chlordane, chlordecone, DBCP, DDT, dicofol, dieldrin, DDT and metabolites, endosulfan, heptachlor/H-epoxide, lindane (gamma-HCH), malathion, methomyl, methoxychlor, oxychlordane, parathion, synthetic pyrethroids, transnonachlor, toxaphene
Industrial chemicals and by-products	Bisphenol – A, polycarbonates, butylhydroxyanisole (BHA), chloro- and bromo-diphenyl, dioxins, furans, nonylphenol, octylphenol, PBDEs, PCBs, pentachlorophenol, penta- to nonylphenols, perchlorate, PFOA, PFOS, p-tert-pentylphenol, phthalates, styrene
Metals	Cadmium, gadolinium, lead, manganese, methyl-mercury, organic-tins (e.g., TBT)

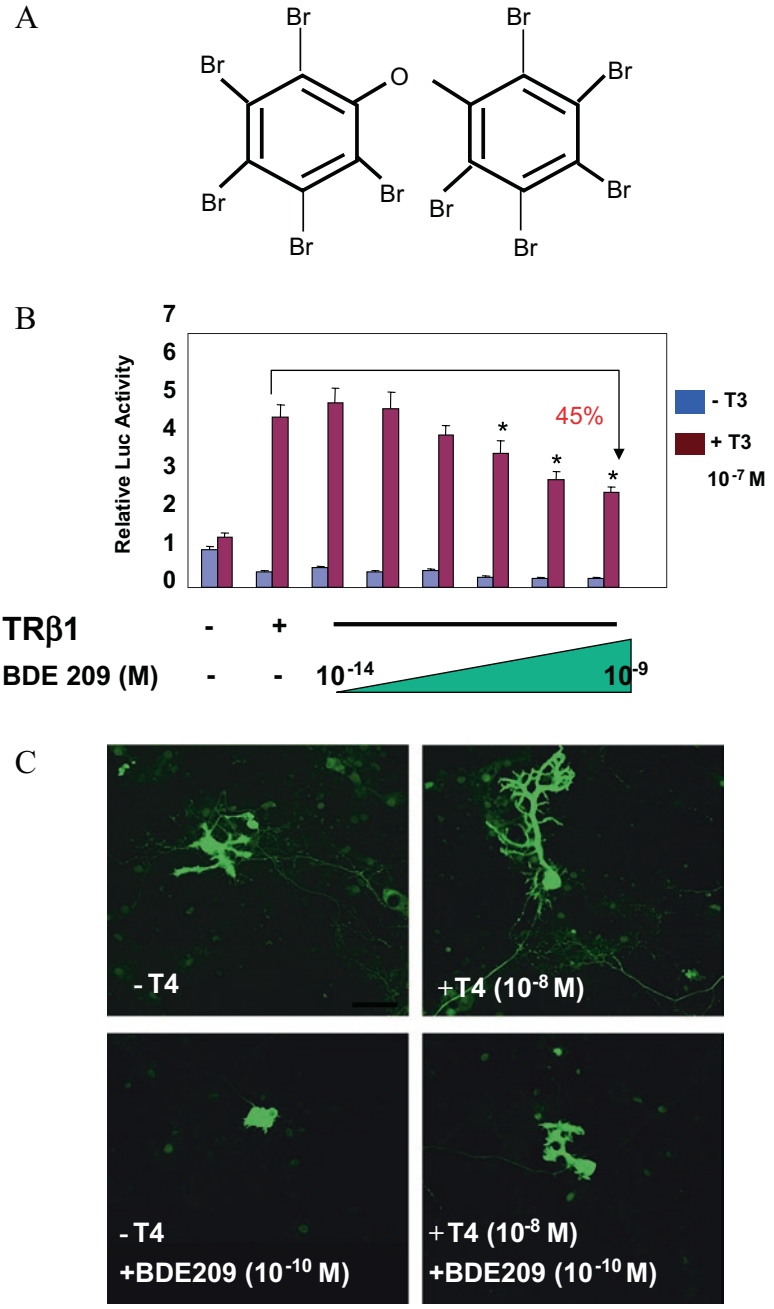

Fig. 7 Representative effect of EDCs (PBDE) on TH-mediated transcription and cerebellar development. (**a**) Chemical structure of BDE209. (**b**) BDE209 suppressed TRβ-mediated transcription, studied by reporter gene assay. *, $p > 0.05$ vs BDE (−) group. (**c**). Effect PBDE (BDE209) on TH-induced Purkinje cell development in primary culture [82]

main reason why such chemicals are not currently defined as EDCs may be that research on EDCs cannot keep up with the increase in newly generated chemicals. Further studies are indeed necessary to identify EDC activity that may cause adverse effect and for the creation of new EDC screening method.

It should be noted that, because concentrations in hormones in plasma are low (nM~pM level), exposure to EDCs, even at low doses, may disrupt hormone action. Furthermore, we do not have the systems to effectively catalyze and excrete most EDCs, because humans are being exposed to EDCs quite recently during the evolutionary process. Thus, EDCs may concentrate in our food chain and accumulate in our body.

So far, 12 chemicals have been identified as being developmental neurotoxic to humans [81]. These are metals and inorganic compounds (arsenic, arsenic compounds, lead, methylmercury, fluoride, and manganese), organic solvents (toluene, tetrachloroethylene), pesticides (chlorpyrifos and DDT/DDE), and industrial chemicals (PCBs and brominated diphenyl ethers [PBDEs]). In cellular or animal study levels, more chemicals may have potential neurotoxic effects [81]. Such chemicals may, at least in part, mediate their action though the endocrine system. In fact, in our previous studies, we have shown that PCBs and PBDEs may disrupt cerebellar development through TH system alterations [15, 82]. Both PCBs and PBDEs inhibit TR-mediated transcription and disrupt TH-induced Purkinje cell development (Fig. 7). Our current study has shown the possibility that several EDCs may affect cerebellar development [15]. Thus, continuous attention should be paid to detect the effect of EDC on cerebellar development. These agents may disrupt cerebellar development even at a low-dose exposure.

Conclusion

Although many hormone receptors are expressed in developing cerebellum, only a limited amount of data is available in this regard. This may a result of the challenges related to research of hormone actions that are mainly mediated by nuclear receptors. Unlike membrane-associated receptors, these act as transcriptional factors to activate or repress the transcription of target genes. Thus, the response is rather slow and various signal transduction cascade may be involved to express their action as a specific phenotype. However, hormonal signaling plays an important role to mediate environmental influences on the developing brain. Thus, hormonal disruptions may cause cerebellar disorders leading to various psychosomatic diseases. It is my hope that this chapter will help increase the understanding of the role of hormones in the developing cerebellum.

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Infections of the Cerebellum

Kevin M. Coombs

Abstract Infectious diseases still account for a significant amount of morbidity and mortality, particularly in developing countries. Although the Lancet's latest *Global Burden of Disease* report indicates life expectancy has increased dramatically during the past decade, partly because of similar dramatic declines in infectious disease-related deaths, estimates are that nearly ten million people die yearly from communicable diseases or from complications arising from prior infection (e.g., liver cirrhosis or liver cancer after hepatitis B and hepatitis C virus infection). Infectious agents include organisms from multiple taxonomic groups and are categorized as bacteria, fungi, viruses, and others. Bacteria and fungi belong to separate taxonomic kingdoms. Viruses are unique and are generally considered to fall outside normal life taxonomy; however, they are, as a group, responsible for more suffering than any other group of infectious agents. Infectious diseases affect every organ system in the body. This chapter will focus on those agents that affect the human central nervous system, with more focus on the cerebellum.

Keywords Virus • Replication • Cerebellar ataxia

General Virology

General Nature of Viruses

Viruses are among the smallest of currently known living organisms. Indeed, there is debate as to whether they should be thought of as alive. Although the idea of a "virus" (Latin for poison to reflect that most can pass through filters known to block bacteria) is only about 100 years old, diseases such as poliomyelitis and rabies

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(discussed more fully below) have been known for millennia. Although we have been aware of viral agents for a relatively short period of time, viruses are probably as old as life itself and appear to have coevolved with most other life forms.

Virus Morphology

Most viruses are very simple in structure. Most consist of both protein and nucleic acid. Some exceptions are viroids, plant pathogens that consist solely of RNA, and prions, agents that may consist only of a misfolded host protein. Some viruses also contain lipid envelopes derived from the host cell in which the virus grew. Viruses exist in two general forms. The form inside an infected cell that is actively replicating may be considered "alive." The mature form of the virus that is passed from one susceptible host to another and that is usually referred to as "virus" is known as the *virion*, which is analogous to a seed or spore. The virion, like a seed, is a stable structure whose primary function is to protect the viral genetic material until it reaches the interior of a suitable host. All viruses are obligate intracellular parasites because they are incapable of growing by themselves.

There is enormous variability in virion size and structure. For example, the smallest currently known animal viruses are the *Parvoviridae* (e.g., human parvovirus B19 which is <20 nm in size) and the largest currently known animal viruses are the *Poxviridae* (e.g., vaccinia virus and the smallpox agent *Variola major* which are approximately 200 × 300 nm in size). Ebolaviruses, members of the *Filoviridae* family, are filamentous with lengths up to 14,000 nm and diameters of only 80 nm [29]. Several viruses, colloquially known as "giant viruses" (e.g., *Mimiviruses* and *Pandoraviruses*) can reach up to 1.5 μm in size [51].

There also is considerable variability in virion complexity. Viruses such as *Parvoviridae* consist of a small nucleic acid surrounded by 60 copies of a single protein. Other viruses, such as the *Papillomaviridae* and the *Adenoviridae*, may be more complex and larger, consisting of a larger piece of nucleic acid and more than 60 copies of multiple proteins. Other viruses, including the *Togaviridae*, *Rhabdoviridae*, *Paramyxoviridae*, *Herpesviridae*, and the human immunodeficiency virus (HIV), which belongs to the family *Retroviridae*, contain a single genome segment and different numbers of various proteins encased in a lipid membrane. A few viruses, such as the *Reoviridae* and the influenza viruses, members of the *Orthomyxoviridae* family, contain different amounts of various proteins and multiple segments of nucleic acid. Finally, some viruses, such as those in the *Nanoviridae* family, have segmented genomes encased in individual capsids. In order for these viruses to successfully replicate, a cell must be infected with multiple particles that collectively provide all the genome segments [77].

With the possible exception of prions, the agents responsible for spongiform encephalopathies, all currently known viruses, contain as their genetic material either DNA or RNA. Most viruses use this genetic information for both *replication*

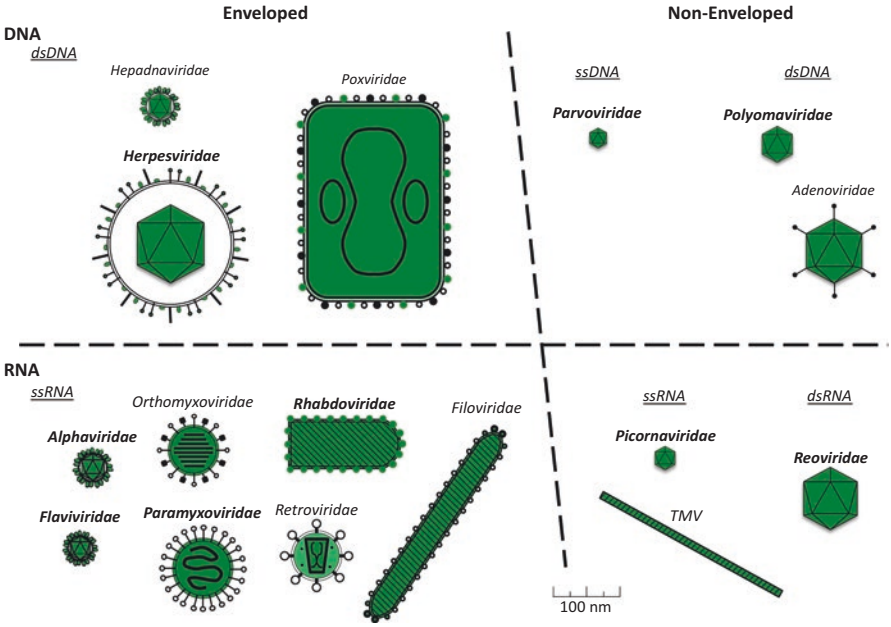

Fig. 1 Diagrammatic representations of selected virions. Viruses are divided according to whether their genomic material is DNA (*top*) or RNA (*bottom*) and whether the capsid is surrounded by an envelope (*left*) or nonenveloped (*right*). Where applicable, each group is further subdivided depending upon whether the nucleic acid is single stranded (*ss*) or double stranded (*ds*). All viruses are shown at approximately the same scale to indicate relative sizes; *bar* at *bottom* represents 100 nm (= 0.1 μm). Virus family names (ending in the suffix *viridae* and italicized) are indicated, with those known to be involved in cerebellar infection bolded and in larger font

and for *transcription*. Replication is the process by which the genetic material is copied into full-length exact genomic replicas that will be packaged into progeny virions (discussed more fully in sections "Herpesviruses" and "Myxoviruses"). Transcription is the generation of messenger RNA (mRNA), whether from DNA or RNA, for production of viral proteins. Thus, a convenient way to classify viruses that directly impacts how (and where) the virus replicates and how it causes pathology is by genomic nucleic acid type (Fig. 1). For example, most DNA viruses will replicate in the host cell's nucleus because this is where enzymes needed for DNA replication and synthesis are located. The *Poxviridae* are exceptions because they encode all their own necessary DNA enzymes. By contrast, most RNA viruses do not require DNA enzymes, so they usually replicate in the cell's cytoplasm. The *Retroviridae* are exceptions because they must replicate through a DNA intermediate so their replication involves the host cell's nucleus. In addition, some other RNA viruses, such as the influenza viruses (*Orthomyxoviridae*), perform some of their replication steps in the cell nucleus because they need to "steal" nascent cellular messenger RNA (mRNA) caps to prime their own transcription.

The viral nucleic acid may also be either double stranded (ds), like cellular DNA genomes, or may be single stranded (ss). If single stranded, the genome also may be of either positive (+) polarity or of negative (−) polarity. By convention, mRNA is considered to be (+) polarity. Therefore, the template DNA or RNA strand that is transcribed to produce mRNA is usually (−) polarity. The important implications of these differences and how they impact taxonomic classification and replication are described more fully below in sections "Virus Classification" and "Virus Replication."

The viral genome may also range in size. The term *genome* refers to all the nucleic acid of a virus, and the term *gene* usually refers to that part that encodes a specific protein. The smallest viruses (e.g., *Parvoviridae*) have genomes of about 5,000 nucleotides (= 5 kilobases or 5 kb) and contain two genes. The largest human viruses (e.g., *Poxviridae* and *Herpesviridae*) can have genomes of 200 kbp or larger and can encode more than 200 proteins. Most viruses have genomes with sizes that are intermediate. For most viruses, all genes are found on a contiguous single linear strand of nucleic acid. Some viruses have circular genomes rather than linear genomes, and a few viruses (e.g., *Reoviridae* and the influenza viruses) have segmented genomes.

Viruses encode proteins that are considered either *structural* or nonstructural. Structural proteins are those found within a virion particle and are usually identified in highly purified viral preparations. There are usually a characteristic, fixed number of structural proteins within any given virion. For example, poliovirus contains a single copy of a VPg protein and 60 copies each of four other proteins named VP1, VP2, VP3, and VP4. However, nonstructural proteins are encoded by the virus and present in infected cells but are not incorporated into the virion.

Some viruses, such as *Human immunodeficiency virus*-1 (HIV-1) (e.g., [37, 70]), herpesviruses [52], filoviruses [78], and influenza viruses [74], also incorporate functional host-derived proteins into the virion. These host proteins may play important roles in the virus life cycle [3, 25, 81].

Collectively, the viral protein and nucleic acid constitute a complex known as the viral *capsid*. Viral capsids are usually of one of two forms. In one form, the viral capsid protein is wrapped along the nucleic acid to create a helical arrangement, the length of which is usually determined by the length of nucleic acid. Examples of this type include tobacco mosaic virus (Fig. 1) and most currently known (−) sense animal RNA viruses, such as the *Orthomyxoviridae*, *Paramyxoviridae*, and *Rhabdoviridae*. Many (−) ssRNA virions have their helical capsids wrapped within a lipid envelope, resulting in an overall spherical virion shape. The second method to surround the nucleic acid with a protein coat is to build a three-dimensional cage, which usually takes the shape of an icosahedron, a 20-sided semispherical structure. There are rigid "rules" for building an icosahedron, and examples of this arrangement include poliovirus and JC virus (Fig. 1). Some viruses (e.g., *Retroviridae*, which have conical capsids and *Poxviridae*, which have ovoid capsids) are exceptions (Fig. 1).

As indicated earlier, some viral capsids are surrounded by a host-derived lipid membrane (envelope). Therefore, the presence or absence of such an envelope is another way to classify viruses (Fig. 1). When an envelope is present, the inner

nucleoprotein structure is called a nucleocapsid. Some viruses, such as the *Orthomyxoviridae* (e.g., influenza virus), the *Paramyxoviridae* (e.g., measles virus), and the *Rhabdoviridae* (e.g., rabies virus), contain nucleocapsids that are helical and that are surrounded by a membrane. Icosahedral nucleocapsids also may be surrounded by an envelope, as in *Flaviviridae* (e.g., *Dengue virus*), *Togaviridae* (e.g., *Chikungunya virus*), and *Herpesviridae* (e.g., herpes simplex viruses). For most enveloped viruses, the lipid membranes are acquired as the nucleocapsid buds through a cellular membrane. Many such viruses pick up this envelope as they pass through the cell's plasma membrane, whereas others pick up their membranes while passing through other internal cellular membranes.

Virus Classification

There are currently >3,500 known virus species organized into >50 families [46]. This list will increase as new viruses are discovered. Several classification strategies have been developed to organize viruses. In addition to organizing viruses according to their overall structure (helical vs icosahedral or otherwise), genetic material (DNA vs RNA, single stranded vs double stranded) and presence or absence of an envelope, another key distinguishing feature is how the genetic material is converted into mRNA. This classification scheme was proposed by Dr. David Baltimore [6] and is therefore known as the "Baltimore scheme" (Fig. 2). Class I viruses contain a dsDNA genome and mRNA is transcribed from the (−) sense DNA strand. Examples of such viruses are the *Herpesviridae* and *Polyomaviridae*. Class II viruses have ssDNA genomes, which are usually (−) sense so their genomes can be transcribed directly into mRNA. Class III viruses (e.g., *Reoviridae*) have dsRNA genomes; mRNA is transcribed from the (−) sense strand. Class IV viruses have (+) ssRNA (e.g., equine encephalitis viruses in the *Alphatogaviridae* family and *Zika virus* in the Flaviviridae family). The viral genome serves directly as mRNA. Class IV viruses (e.g., *Rhabdoviridae* and *Paramyxoviridae*) possess (−) ssRNA that is transcribed into mRNA by a viral-encoded RNA-dependent RNA polymerase that must come into the cell as part of the infecting virus. Class VI viruses (e.g., the retrovirus HIV) contain (+) ssRNA genomes that initially needs to be converted into dsDNA by a viral-encoded reverse transcriptase. Class VII viruses (e.g., *Hepadnaviridae*) have a partial dsDNA genome that is replicated through an RNA intermediate by a viral-encoded reverse transcriptase.

The type of viral nucleic acid, as distinguished by Baltimore classification, also has implications for how the viral genomes are replicated. Class I viruses use their dsDNA genomes as a template to synthesize more dsDNA during genome replication. The Class VII *Hepadnaviridae* are an exception because replication involves a reverse transcriptase-generated RNA intermediate that is longer than the DNA genome. Class II viruses use dsDNA replicative intermediates to copy their genomes. For Class III viruses, the mRNA that was used for protein synthesis is then copied by viral enzymes into a (−) sense RNA that remains associated with the mRNA

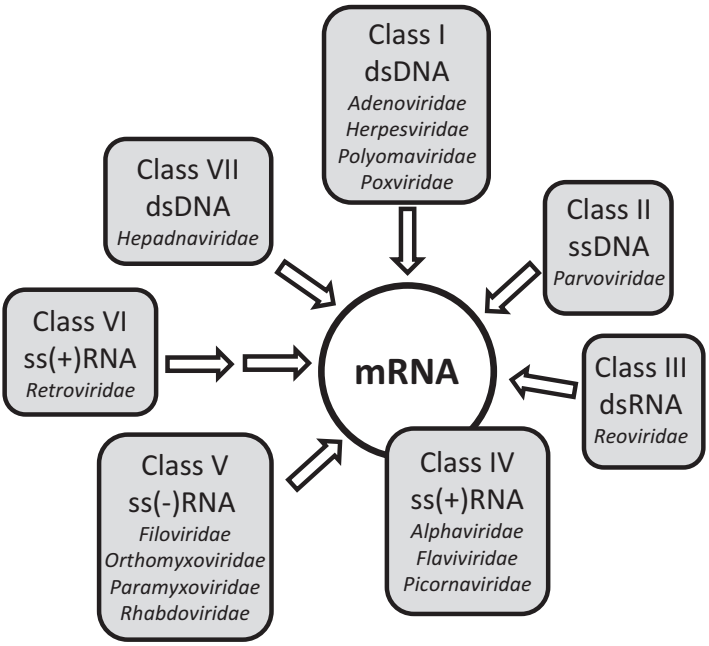

Fig. 2 The Baltimore transcription scheme organizes viruses according to their genomic nucleic acid content and the strategies they use to produce mRNA. By definition, mRNA is (+) sense. Note that Class IV viruses have (+)RNA that serves directly as mRNA. Class VI retroviruses undergo reverse-transcription to convert RNA into DNA, which is then used to transcribe mRNA

template to regenerate progeny dsRNA. Class IV viruses with (+) ssRNA genomes produce a full-length (−) ssRNA intermediate that is used as template for more genomic (+) ssRNA. Class V viruses use the same strategy for genomic replication as class IV viruses, except they start with a (−) ssRNA genome and use a (+) ssRNA strand as an intermediate. For the class VI *Retroviridae*, (+) ssRNA genomes are copied by a unique mechanism. The (+) ssRNA is copied into (−) ssDNA, which then serves as a template to copy a (+)ssDNA strand. This dsDNA molecule is transcribed into mRNA, one of which (a non-spliced form) serves as progeny genome.

Virus Replication

The ways in which a virus can enter a cell and subvert the cell to create progeny virus copies is a complex process that is investigated using numerous approaches including traditional virology and system biology. Advances in gene sequencing and bioinformatics, combined with such approaches as mass spectrometry-based proteomics, RNA micro-arrays, and next-generation sequencing, have led to a better understanding of virus-host interactions during the process of virus replication.

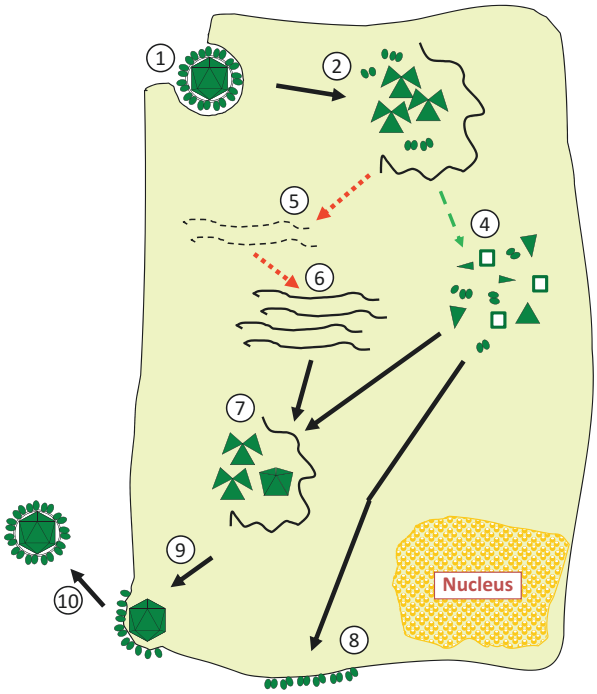

Fig. 3 Overview of a typical enveloped RNA virus replication cycle. Steps are (*1*) attachment and entry; (*2*) uncoating and release of genomic material; (*3*) transcription of genes (not shown in this figure because Class IV RNA virus genomes serve directly as mRNA – see Fig. 2); (*4*) translation of mRNA and production of viral proteins; (*5*) first replication step, in which (+) sense incoming genomic material is converted to (−) sense template; (*6*) second replication step in which (−) sense template is recopied into many (+) sense progeny genomes; (*7*) aggregation of progeny capsid proteins with progeny genomes; (*8*) transport of viral envelope proteins through Golgi apparatus to cellular membranes; (*9*) association of progeny nucleocapsids with envelope proteins; and (*10*) exit from cell by budding. Wavy lines represent viral nucleic acid, small filled ovals and triangles represent viral structural proteins that assemble into complexes, and small open squares represent nonstructural proteins that are present within the cell and assist in viral replication and assembly but are not found within mature virions

Virus replication can occur only in a live cell because, as indicated earlier, viruses are obligate intracellular parasites. Despite some differences in some details of virus replication, as suggested by the diversity within the Baltimore scheme (see above), most viruses share several common features. There is a general flow of events that occur during viral replication for most viruses (Fig. 3). (1) To start an infection, the virus must attach to and enter a host cell. This interaction is specific and involves both viral proteins and a host cell surface component. The virus' host range (described below) determines in large part whether a virus can attach to any given host cell. Some viruses, such as HIV and hepatitis B virus, interact with highly specific host cell components, whether carbohydrates, proteins, or glycolipids, whereas others, such as mosquito-vectored arboviruses like *Yellow Fever virus* and *Zika virus*, both

members of the *Flaviviridae*, can interact with more ubiquitous components found on both vertebrate and invertebrate cells. (2) Once a virus has entered the cell, it must fall apart (uncoat) to allow the incoming viral genetic information to be acted upon by host enzymes. Uncoating may occur at the plasma membrane during entry (e.g., paramyxoviruses), inside the endosome (e.g., adenoviruses and orthomyxoviruses), in the cytoplasm, or at the nuclear membrane (e.g., herpesviruses). For many viruses, the genome is completely uncoated to allow unrestricted access to the nucleic acid (e.g., picornaviruses). For many others (e.g., (−) ssRNA viruses like the *Paramyxoviridae* measles virus) and the dsRNA *Reoviridae*, the viral genome is only partially uncoated, with remaining viral proteins serving enzymatic functions.

The greatest variability in viral life cycles occurs during intermediate replication stages. Events that take place during this time are (3) transcription (the process whereby the nucleic acid, whether RNA or DNA, is copied to produce complementary positive sense mRNA) (for viruses in Class IV (e.g., *Alphatogaviridae* [depicted in Fig. 3], *Flaviviridae* and *Picornaviridae*), the genome itself serves as mRNA, so this initial transcription is not necessary), (4) translation ("reading" of the mRNA nucleotide sequence by cellular ribosomes to produce viral proteins), and (5) replication (the copying of parental genomic material that serves as the template to produce an identical copy). For many viruses, this involves a few steps. As depicted in Fig. 3, the incoming genome is first copied into (−) sense template, which is then used (6) to make multiple (+) sense genomes. The progeny genomes aggregate with newly made viral capsid proteins (Step 7), whereas viral envelope proteins (if the virus is enveloped) are shuttled through the cellular endoplasmic reticulum and Golgi apparatus (Step 8) on their way to the plasma membrane. (9) After progeny nucleocapsids are produced, they migrate to the plasma membrane where they bind to the envelope proteins and (10) bud out of the cell. Budding may or may not damage the cell, and in some cases, the cell will recover from the viral infection. Budding may occur at either the apical (top) surface (e.g., orthomyxoviruses and paramyxoviruses) or at the basal (bottom) surface (e.g., rhabdoviruses and some retroviruses) or may not be restricted to a specific cell surface.

Cell Tropism

Viruses parasitize every type of living organism, from bacteria to plants and animals and, in some cases, even other viruses [49]. A small number of viruses can infect organisms in multiple different Kingdoms. For example, members of the *Rhabdoviridae* family are capable of infecting plants and animals, although any given virus usually is limited to one kingdom or the other. Most currently known viruses infect a small range of host types. This is known as *cell tropism*. For example, a virus that infects a particular bacterial species usually cannot infect all bacteria and normally also cannot infect any animals or plants. Similarly a typical plant virus can infect some, but not all, plant species but cannot infect animals or bacteria. Likewise, most animal viruses are limited to infecting only certain animal species.

There also is considerable variability in cell tropism within the above kingdoms. Some viruses (e.g., HIV) are very limited in their cell tropism, capable of infecting only a limited type of human-derived cell (e.g., CD4+ cells). However, a few viruses (e.g., arthropod-borne *Flaviviridae*) can infect vertebrate animals of diverse orders (horses and humans), of different classes (birds and humans), and of different Phyla (animals and the insects that vector the virus from one animal host to another).

The basis for cell tropism depends upon whether a particular virus can enter and replicate in a cell. In many cases, this is determined by whether the virus can recognize and enter the host cell (see above). Thus, viruses like the *Flaviviridae* that can infect a wide range of cells usually recognize a ubiquitous cell surface receptor, whereas viruses like HIV that are restricted to a small number of cells recognize a highly specific cell molecule. Cell tropism may also depend upon the intracellular milieu. If key host molecules that are required for virus replication are not present, the infection may be abortive.

In addition to cell tropism, which limits the type of host organism (e.g., animal, bacterium or plant) a virus can infect, many viruses can only infect particular tissues within a susceptible organism. This is known as *tissue tropism*. For example, herpesviruses generally can replicate in many tissue types (brain, liver, skin, etc.), whereas hepatitis viruses can replicate efficiently only in liver tissue. As is the case for cell tropism, cellular parameters (cell surface molecules and presence or absence of key intracellular enzymes) determine the basis for tissue tropism. Subsequent sections of this chapter will focus on viruses with a tropism for the brain and cerebellum.

Virus Infections of the Brain and Cerebellum

A variety of infectious agents attack the human central nervous system. One of the best-known viruses, which has a case fatality rate of 100% if untreated, is rabies virus, a member of the *Rhabdoviridae* family. Once the virus reaches the CNS after being injected by the bite of an infected animal, or, in rare cases after organ transplantation from infected donors [53], rabies primarily replicates in dorsal root ganglia, motor neurons, and sensory neurons in the spinal cord [24]. Viral antigens have been detected within the cerebellum shortly after infection [53], but it remains unclear whether the cerebellum plays a major role in disease propagation or pathogenesis.

Enteroviruses

Another of the best-known viruses that has long been associated with neurologic manifestations is polio virus. Poliomyelitis has been recognized as a disease for millennia. This virus is a member of the *Picornaviridae* family, Enterovirus subfamily. Enteroviruses are transmitted by the fecal-oral route. If ingested, the virus initially replicates in intestinal tissues. In some cases the virus travels to the CNS. Severe manifestations are relatively rare, occurring in less than 2% of infections, with most infections being asymptomatic or mild. When serious, CNS

involvement and paralysis can occur. There have been reports of cerebellar ataxia associated with poliovirus infection [18]. Cerebral ataxia affects muscle coordination and leads to gait and posture irregularities, lack of fine motor coordination, cognitive and mood problems, increased fatigue, speech difficulties, and visual abnormalities.

Other members of this virus family also are responsible for cerebellar infections and clinical manifestations. Enterovirus 71 is estimated to have caused millions of infections during the past decade [55] and, after *Listeria* (see below), may be the second most common agent responsible for serious brainstem encephalitis (rhombencephalitis). Complications range from self-limiting aseptic meningitis to acute flaccid paralysis that mimics poliomyelitis and overwhelming brainstem encephalitis. Coxsackie virus has also been associated with cerebellar FDG-PET hyperactivity [62].

Arboviruses

A large number of different arboviruses (arthropod-borne viruses) are associated with neurological infections that include the cerebellum (reviewed in [71]). Arthropod-borne agents fall into several taxonomic groups, and among viruses, arboviruses exist within at least five families [39]. The principle families are *Alphatogaviruses* (various equine encephalitis viruses including Eastern, Western, Venezuelan, and *Chikungunya virus*) and Flaviviruses (e.g., Dengue, Japanese encephalitis, West Nile, and Zika), which contain ss(+)RNA genomes. Other ss(+) RNA arboviruses that infect humans belong to the *Bunyaviridae* (e.g., Rift valley fever virus and Congo-Crimean hemorrhagic fever virus). In addition, members of the dsRNA *Reoviridae* (e.g., Colorado tick fever virus) and large dsDNA *Asfarviridae* (e.g., African swine fever virus) are arboviruses.

Alphatogaviridae

Rubella virus, the agent responsible for German measles, has been associated with cerebellar abnormalities [50]. A large outbreak of Venezuelan equine encephalitis virus (VEEV) in Columbia and Venezuela in 1995 resulted in >300 hospitalizations, and more than half the patients had neurologic complication including cerebellitis [58]. *Chikungunya virus* has also been associated with cerebellar ataxia [42].

Flaviviridae

Although dengue virus is rarely associated with CNS involvement, infection with dengue has led to cerebellar manifestations. In one report, a Sri Lankan male presented with thrombocytopenia but subsequently developed ataxia and magnetic

resonance imaging (MRI)-confirmed cerebellitis; serology suggested a *Dengue virus* and *Epstein-Barr virus* coinfection [43]. In addition, outbreaks of dengue fever in Sri Lanka involving tens of thousands of cases presented with acute cerebellar syndrome [91, 92]. Magnetic resonance imaging of eight encephalitic cases in India showed cerebellar involvement in all eight [36]. West Nile virus infection is also associated with neurological abnormalities in multiple cerebral compartments including the cerebellum [21]. Deer tick virus caused an extensive necrotizing meningoencephalitis that involved numerous brain regions including the cerebellum in a fatal encephalitis case [86]. The newly re-emerging *Zika virus* has been strongly associated with microcephaly, and these brain malformations also include the cerebellum [22, 57, 68, 82, 89].

Herpesviruses

Several different viruses belong to this family. The best-known may be Herpes simplex virus type 1, which is associated with oral cold sores. These viruses are notable for their capacity to go latent after an acute infection and to then "hide" within the host, being reactivated months to years later. The family is subdivided into three major groups: alphaherpesviruses, which primarily infect a wide range of cells, can establish latency in neurons and have relatively rapid replication kinetics; betaherpesviruses, which replicate more slowly and primarily establish latency in leukocytes; and gammaherpesviruses, which have variable infection kinetics and usually have replication restricted to lymphoid cells and establish latency in cells of the immune system, although other cell types can be infected. *Epstein-Barr virus*, a gammaherpesvirus, which is associated with infectious mononucleosis, Burkett's lymphoma and nasopharyngeal carcinoma, causes acute postinfectious cerebellar ataxia (APCA) [1, 15, 72]. The alphaherpesvirus Varicella zoster virus, which causes chickenpox/zoster, infects the cerebellum and has been associated with cerebellar ataxia, segmental brainstem myelitis, polyneuritis, and vasculopathy [10, 32, 60, 67, 69, 75].

Myxoviruses

There are several myxoviruses, belonging to multiple virus families that infect the cerebellum. Myxoviruses are so named because they are primarily respiratory. Two major families are the *Orthomyxoviridae* (e.g., influenza virus) and *Paramyxoviridae* (e.g. measles virus). Influenza viruses of the H1N1 and H3N2 subtypes, the subtypes responsible for the past few pandemics, have also been associated with encephalitis involving the brainstem and cerebellum [23].

Several paramyxoviruses have also been found to infect or affect the cerebellum. These include measles virus, mumps virus, and respiratory syncytial virus.

Respiratory syncytial virus was associated with cerebellar hemispheric cortical edema that involved ataxia and hypotonia [85].

Prions

The agents that cause spongiform encephalopathies belong to a unique group, and there is debate as to whether they should be considered viruses. However, they certainly are not bacteria or fungi and so will be discussed here. There are several such agents, responsible for a variety of diseases, including Kuru, iatrogenic Creutzfeldt-Jakob disease, and bovine spongiform encephalopathy-induced variant Creutzfeldt-Jakob disease. Abnormal prion protein deposition has been observed in Creutzfeldt-Jakob disease patients [30], other patients presented with cerebral cortical hyperintensity [40], and a recent quantitative proteomic screen identified substantial dysregulation of s-nitrosylated proteins within the cerebellum of prion-induced Creutzfeldt-Jakob disease patients [12].

Other Viral Agents

In addition to the virus agents mentioned above, a few other viruses have been shown to infect and/or affect the cerebellum. Many cause APCA. These include rotavirus (family *Reoviridae*) [45, 48, 87], JC virus (family *Polyomaviridae*) [20, 31, 84], parvovirus B19 [33, 34, 76, 88], and possibly adenovirus [64, 83]. Mild cerebellar signs were also seen in an Ebola virus-infected patient [14].

Bacterial and Fungal Infections of the Cerebellum

In addition to viruses, there are other infectious agents that affect the cerebellum.

Bacteria

Several bacteria infect the cerebellum. *Listeria monocytogenes* may be the most common infectious cause of serious brainstem encephalitis (rhombencephalitis), including cerebellar involvement [11, 16, 66, 73]. These infections and cerebellar involvement have potential devastating life-threatening consequences; thus, Pruitt indicates that suspected cases should be treated empirically with ampicillin pending culture confirmation [67].

Mycoplasma pneumoniae has also been implicated in cerebellar ataxia [9, 35, 63]. A large-scale analysis of more than 790 patients identified large numbers of

neuropsychiatric manifestations after infection by *Salmonella typhi*, the etiologic cause of typhoid fever, including eight cases of cerebellitis [2]. *S. typhi* infection also leads to T1-weighted MRI hypointense cerebellar regions [61]. Infection and resulting cerebellar complications continue to occur, although antibiotic treatment of a few cases led to complete recovery [38].

Borrelia burgdorferi, the causative agents of Lyme disease, have also been associated with cerebellar ataxia and with abnormal MRI lesions [4] and PET images [41] (reviewed in [28]). The bacterium *Tropheryma whipplei*, which causes Whipple's disease, has also been found to cause abnormal cerebellar MRI and ataxia [17, 54, 56].

In addition to the capacity of live infectious agents to cause cerebellar ataxia, it has been recognized that vaccinations against various agents also may cause ataxia. The best-known examples are vaccination with DPT (diphtheria-pertussis-tetanus vaccine) [7, 44, 47, 59]. A recent report also identified cerebellar ataxia after vaccination with meningococcal group C agents [19].

Fungi

Several fungal species, including *Aspergillus* [13, 26, 27], *Histoplasmosis* [79, 90], *Candida* [80], *Exserohilum rostratum* [8], and *Phialemonium* [5], have also been associated with cerebellar infection [65, 67].

In conclusion, the cerebellum is susceptible to infection by a large number of bacterial, fungal, and viral agents, and these infections can have devastating consequences.

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Neuroimmune Mechanisms of Cerebellar Development and Its Developmental Disorders: Bidirectional Link Between the Immune System and Nervous System

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Abstract Understanding the cross talk between the immune system and cerebellum development has noticeable implications for understanding and management of neurodevelopmental disorders. Our knowledge about cerebellar developmental maturation and remodeling is improving. Immune cells have different functions in a healthy state, but those functions are compromised during developmental stages in mammals. In this chapter, we highlight the evidence that indicates an important role of the immune system within the cerebellum and brain. We discuss the contribution of different immune responses in the development of the cerebellum and its associated disorders and highlight current understanding of the mechanisms and insights involved in these processes. Immune pathways that have a crucial role in cerebellar development are likely to become therapeutic targets for several neurodevelopmental disorders. Therefore, this information may suggest new therapeutic approaches

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to developmental disorders of the cerebellum through suppression or activation of selected immune pathways.

Keywords Cerebellum • Brain • Innate immunity • Adaptive immunity • Cytokines • Hypothalamic–pituitary–adrenal

Abbreviations

AICA	Anterior inferior cerebellar artery
ALRs	AIM2-like receptors
ALS	Amyotrophic lateral sclerosis
ANS	Autonomic nervous system
APCs	Antigen-presenting cells
BBB	Blood–brain barrier
CCL	C-C motif chemokine ligand
CNS	Central nervous system
Cop-1	Copolymer 1
CSF	Cerebrospinal fluid
DAMPs	Damage-associated molecular patterns
DC	Dendritic cells
EAE	Experimental autoimmune encephalomyelitis
EGL	External granule cell layer
FOXP3	Forkhead box P3
GAD	Glutamic acid decarboxylase antibodies
GIT	Gastrointestinal tract
HE	Hashimoto's encephalopathy
HSP	Heat shock proteins
IBS	Irritable bowel syndrome
IFN	Interferon
Ig	Immunoglobulin
IGL	Internal granule cell layer
IL	Interleukin
LGP2	Laboratory of genetics and physiology 2
MDA5	Melanoma differentiation-associated gene 5
MHC	Major histocompatibility
MIP	Macrophage inflammatory protein
MSA	Multiple system atrophy
NLRs	Nod-like receptors
OPCA	Olivopontocerebellar
P2X7R	Purinergic receptor P2X7
PACA	Primary autoimmune cerebellar ataxia
PAMPs	Pathogen-associated molecular patterns
PICA	Posterior inferior cerebellar artery

PRRs	Pattern recognition receptors
(RAG)-1	Recombination activating gene
Rig1	Retinoic acid-inducible gene-1
RLRs	RIG-like receptors
Rora	Retinoic acid-related orphan receptor alpha
ROS	Reactive oxygen species
SCA	Superior cerebellar artery
SCID	Severe combined immunodeficiency
SND	Striatonigral
SOCS3	Suppressor of cytokine signaling 3
TGF	Tumor growth factor
Th	T helper
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
Treg	Regulatory T cells
URL	Upper rhombic lip

Introduction

The cerebellum is an important motor and non-motor structure in the central nervous system (CNS), it includes more neurons than the entire cerebral cortex [1], and it is well-conserved across evolution. Despite its prominent architecture and some clear syndromes related to its malfunction, the exact role of immune regulation during cerebellum development has not been well studied. Thus, many researchers are investigating the role of the immune system within the cerebellum.

Over the past few decades, the functional autonomy of both the immune system and the CNS has been successfully challenged, and innovations in the field of neuroimmunology and psychoneuroimmunology have revealed that the immune system and the CNS are closely related and function together [2, 3]. There is a growing understanding that neurodevelopmental disorders are related to cerebellar deficits, but molecular mechanisms and underlying pathways of cerebellar defects remain poorly understood [4]. Developmental studies have revealed that the cerebellum evolves in successive waves of progenitor proliferation/migration throughout the embryonic and postnatal phases, which may identify new therapeutic options.

The immune system can interact with the CNS through immune mediators such as primary cytokines, CNS-derived cytokines, neurotransmitters, and neuropeptides secreted by different immune cells [5]. Bidirectional communication between immune responses and the CNS and their effect on cerebellar development has not been well studied, but direct bidirectional projections between the cerebellum and hypothalamus and the cerebellohypothalamic and hypothalamocerebellar projections have been demonstrated [6–8]. Stimulation of the cerebellar fastigial nuclei induces a postsynaptic response or an alteration in unitary activity via cerebellohypothalamic projections in the hypothalamus [9], demonstrating that the cerebellum can indirectly influence immune cell function through cerebellohypothalamic

projections. In this chapter, we will review possible direct or indirect interrelations between the immune system and cerebellum and how the immune system can affect the development of the cerebellum to maintain a homeostatic state or regulate pathological conditions such as cerebellum developmental disorders.

Anatomy of the Cerebellum and Interconnection with Other Central Centers Implicated in Neuroimmune Regulation

The anatomical features of the cerebellum, described in chapter "The Embryology and Anatomy of the Cerebellum", are relevant to understand how immune and inflammatory responses are generated and how these immune responses could affect cerebellar development. The hypothalamus also exerts specific neuromodulation on the cerebellum, which could impact the immune response. This modulation occurs because the cerebellar cortex receives two well-identified types of afferent fibers: mossy fibers and climbing fibers. There is a third type of afferent, the neuromodulatory fiber that consists of characteristically beaded fibers, which contain amines or neuropeptides [10, 11]. For example, histamine-containing fibers originate from the tuberomammillary nucleus of the hypothalamus and broadly spread into the cerebellum [11]. Moreover, beaded fibers containing angiotensin II result from the paraventricular and supraoptic nuclei of the hypothalamus [12] and impact comprehensively upon the cerebellum.

The relationship between circulating hormones (thyroid hormones, sex hormones) and cerebellar development is well studied (see chapter "Hormonal Regulation of Cerebellar Development and Its Disorders"). These hormones have immunomodulatory effects and can shape different immune responses. Thyroid hormone and its receptor, which is a ligand-regulated transcription factor binding to a specific DNA sequence called thyroid hormone-responsive element, have a particularly vital role in brain development [13]. The receptor recruits coactivators and corepressors in a ligand-dependent manner to regulate the transcription of target genes. It may also interact with other nuclear receptors such as retinoic acid-related orphan receptor alpha (Rora), whose expression is regulated by the thyroid hormone during the first two postnatal weeks. In perinatal hypothyroidism, Purkinje cell dendrites have greatly reduced growth and branching with a reduction of synapses between granule cells and Purkinje neurons, which is associated with delayed migration of granule cells to the internal granule cell layer and deficient synaptic connectivity within the cerebellar cortex [14]. Experimentally, thyroid-deficient rats show a persistence of climbing fiber synaptic sites for a longer time along with an underdevelopment of cerebellar glomeruli [15]. These effects could be attributed to hyperthyroidism, which reduces the pro-inflammatory properties of monocytes and macrophages and promotes phagocytosis, and there may also be elevated levels of reactive oxygen species (ROS) during hypothyroidism [16]. A better understanding of the links between such hormones and immune responses could provide new insights toward clarifying the potential effects of several immune responses on the development of the cerebellum.

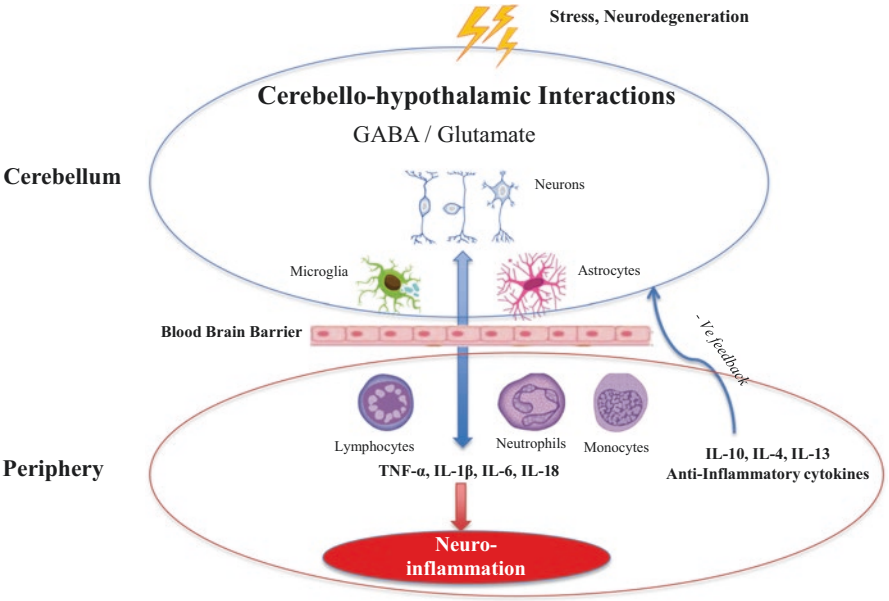

Fig. 1 Simplified illustration shows the interaction between cerebellohypothalamic projection, neurons, astrocytes, microglia, and peripheral immune responses. In pathological conditions, there is a cerebellohypothalamic interactions via the balance between GABA and glutamate to activate neurons. Neurons deliver damage signals to glial cells and astrocytes to interrupt blood–brain barrier that lead to activate innate and adaptive immune responses and pro-inflammatory cytokine productions and end with neuro-inflammation. Anti-inflammatory cytokines result in deactivation of glial cells and astrocytes and in turn maintain homeostasis

Cerebellar immunomodulation exists, and it may be regulated by the hypothalamus, but anatomically, there is no direct connection between the cerebellum and the immune system. The cerebellum communicates with the immune system through the cerebellohypothalamic projections, which are direct projections from the cerebellum to the hypothalamus, and this pathway may serve as an important mediator in immune system modulation (Fig. 1). Moreover, many neuropeptides can be released from the CNS and can impact the immune system, which in turn affect the cerebellum, especially in the developmental stages. Thus, various immune responses can shape the development and functional consequences of the cerebellum. However, there are few direct or indirect data that demonstrate these mechanisms.

The Immune System in the Cerebellum

Alterations in immune responses during prenatal or early postnatal development contribute to cerebellar development and disorders. The immune system is designed to reflect surrounding changes and to predict future changes as a defensive mechanism. Communication between the CNS/cerebellum and the immune system is

bidirectional, and both systems shape the other's responses through different mechanisms and mediators. As shown in Fig. 1, the CNS can mediate the innate and adaptive immune responses, which leads to the production of cytokines and which can alter cerebellar development and function. Moreover, pattern recognition receptors and innate and adaptive immune responses play a major role in regulation of the immune system.

Pattern Recognition Receptors

The innate immune system senses commensal and potential antigens and detects tissue disruptions by different receptor types, which are called pattern recognition receptors (PRRs) [17]. PRRs recognize pathogens through specific pathogen-associated molecular patterns (PAMPs) that are expressed by microbes, and they play a major role in sterile inflammation response to endogenous stimuli, called damage-associated molecular patterns (DAMPs). PRRs are among the first responders to cerebellar disorders [18], and activation of these receptors on microglia, neurons, and astrocytes initiates an innate immune response [19]. Microglia, the brain's main resident immune cells, are important to the inflammatory response and can be activated by distress signals released from neighboring cells [20]. Microglia that enter a pro-inflammatory state are mostly activated by PRRs, and they are fundamental components of host innate immunity. Therefore, activation of PRRs results in inflammatory mediator release that helps to remove antigens or restore tissue homeostasis. However, chronic or continuous activation of these receptors can cause inflammatory disorders that impact cerebellar development and contribute to pathogenesis of its developmental disorders.

Toll-like receptors (TLRs) have been characterized in response to pathogens, but they also play a central role in regulating sterile inflammation [21]. TLR4 is a major route of such amplification that may occur via the purinergic receptor P2X7 (P2X7R) [22]. Interleukin-1β (IL-1β) is now counted as a master regulator of neuroinflammation; it leads to a critical contribution to cellular activation and cytokine production [23] and contributes to the pathogenesis of cerebellum disorders, as well as to other acute and chronic diseases of both the peripheral nervous system and CNS [24]. Many DAMPs such as heat shock proteins (HSP60 and HSP70), degradation products of the ECM (hyaluronic acid, fibronectin), and nucleic acids such as mRNA and miRNAs are released passively from necrotic cells after cerebellum injury [25–27]. Mitochondrial DNA and proteins are also considered to be DAMPs, particularly mtDNA and N-formyl peptides [28].

Most cells in the CNS express TLRs, but microglia express the full repertoire of TLRs, which enhance their ability to monitor the CNS and act as the first line of defense [29]. In the cerebellum, microglial activation can be partially explained by aberrant expression of TLRs in the brain regions that are involved in striatonigral

(SND) or olivopontocerebellar (OPCA) [30] and could promote neurodegenerative disorders through amplification of pro-inflammatory cytokines release via alterations in TLRs signaling, which lead to mitochondrial dysfunction that end with excessive reactive oxygen species production [30]. Astrocytes, neurons, and oligodendrocytes also express TLRs in both physiological and pathological cerebellum states [31]. Astrocytes express TLR3 under resting and activated conditions [32] and may elevate TLR2 and TLR4 upon activation [33]. Changes in TLR expression demonstrate their critical role in mediating complex and interconnected processes that are implicated in the development of the cerebellum and its developmental disorders.

NLRs are primarily dedicated to sensing and detecting pathogens, but they have been shown to contribute to the inflammatory responses caused by cerebellar disorders [34]. NLRs are known for their ability to form inflammasomes. Inflammasomes are large multiprotein complexes that activate caspase-1, which is essential for maturation of pro-IL-1β and pro-IL-18 [35] and for programmed cell death [36]. Within the CNS, three different NLR inflammasomes have been described: NLRP1 [37], NLRP2 [38], and NLRP3 [39]. NLRP1 has been defined as mediating the innate immune response after brain disorders [40]. Inflammasomes are present in astrocytes [41] and microglia [42], and neuronal inflammasomes seem to contribute to cerebellum disorders [43]. IL-1β and IL-18 have a crucial role in mediating neuroinflammation and neurodegeneration in the CNS [44]. Experimentally, IL-1β is activated specifically in the cerebellum by the systemic administration of kainate, and it is involved in kainate-induced ataxia in mice. Moreover, IL-18 in the cerebellum is implicated in the recovery phase of kainate-induced ataxia by counteracting the function of IL-1β in the cerebellum [44]. IL-1β also participates in neurological processes and appears to have a role in autism as a mediator of this cerebellar developmental disorder [45]. Homeostatic levels of IL-1β and its antagonist IL-1ra are necessary for proper brain development and function.

Many PRRs that are expressed in the cerebellum can identify pathogenic microbes and are mediated through RLR and ALR [46]. RLRs are cytoplasmic PRRs that detect RNA viruses associated with the production of type I interferons (IFNs) [47]. Two ALRs have been described: IFI16 and AIM2 [48]. In neurons, AIM2 forms an inflammasome that activates pyroptosis, a novel but potentially important mode of cell death [49]. Moreover, scavenger receptors (type A and B receptors) are PRRs that are implicated in the metabolism of cholesterol and lipids and are expressed on microglia, endothelia, and astrocytes [50]. Programmed cell death plays a significant role in the cerebellum development and developmental disorders by affecting the neurons and glia during nervous system development, plasticity, and aging. Thus, defects in this mechanism can impact cerebellum development, which could result in developmental disorders. To summarize, PRRs are among the first responders in the cerebellum, and their activation will trigger an innate immune response.

Innate Immune Responses in the Cerebellum

Immune responses in the cerebellum are a critical component of immune privilege in the CNS, and they are mediated by resident microglia and astrocytes without direct counterparts in the peripheral immune response. Moreover, microglia, dendritic cells (DCs), and astrocytes are also implicated in significant cross talk between CNS-infiltrating T cells, neutrophil complement, and other components of the immune system.

DCs play a critical role within the innate system as antigen-presenting cells (APCs) that induce adaptive immunity. However, there is no evidence that DCs with these abilities exist within the healthy cerebellum or CNS parenchyma. Additionally, some cells express DC surface markers (CD11b, CD11c) in the meningeal CNS covering and in the choroid plexus where CSF synthesis takes place [51]. The nonexistence of parenchymal DCs and the fact that no other parenchymal CNS cells correspond to the functional definition of DCs (e.g., APCs) establish the cellular basis of cerebellum immune privilege. Cerebellum immune tissue is privileged because of its robust intrathecal inflammatory reactions that can damage delicate post-mitotic cells such as neurons and oligodendrocytes. The absence of adaptive immune responses might confer a physiological advantage to the cerebellum. Because antigen entrance into the cerebellum suggests that there is a passage from a peripheral site of entry to the draining lymph nodes or spleen, it would likely be unnecessary for the cerebellum to generate a de novo adaptive immune response. However, further studies are required to support this hypothesis.

The main function of the blood–brain barrier (BBB) is to provide an accurate calibrated chemical and ionic environment to optimize neuronal function and to prevent inflammation by excluding plasma proteins and peripherally derived innate and adaptive immune responses [52, 53]. The parenchymal cerebellum environment has anti-inflammatory proprieties because of high local levels of inflammation-suppressive cytokines (TGF-β, IL-10), and it is supplied with gangliosides, which can be detrimental and lethal to T cells [54, 55]. Moreover, the absence of CNS innate immune cells activating adaptive immunity within the lymphoid organs suggests that resident innate immune cells need to interact directly with the damaged tissue [19].

Microglia are the resident macrophages of CNS. They play critical roles during pathophysiological conditions and display different topographical morphologies across the CNS and during phases of their lifespan [56]. Microglia are implicated in brain development, including in growth of neurites, synaptic pruning, spinogenesis, and apoptosis [57, 58] in areas such as the visual cortex, hippocampus, and retinogeniculate system [59]. However, there are few studies dedicated to the role of microglia during postnatal development. In the cerebellum, microglia are dispersed in both gray and white matter across diverse species, and there is a distinct arrangement of microglial processes according to their location in the cerebellar cortex [60]. Recent findings showed a continuous process of microglial maturation and a nonuniform distribution in the cerebellar cortex, demonstrating that microglia are an essential cellular component of the cerebellum [61]. This has been confirmed in vitro, where the microglia can promote apoptosis of Purkinje neurons [62]. However, there is no information about the presence of this mechanism in vivo.

Microglia also regulate synapse formation and plasticity by phagocytosis of unwanted synapses opsonized with complement components [63]. Impaired phagocytosis leads to an increase in the buildup of cellular debris and has detrimental effects on surrounding neurons, which are suspected to play a role in several neurodegenerative and neurodevelopmental disorders [64].

Neutrophils are an important component of innate immunity and are also considered to be a first line of defense against bacteria, as demonstrated by life-threatening conditions that result from neutrophil deficiency [65]. Neutrophils respond to PAMPs and DAMPs through TLRs and NLRs to increase CD15, CD11b, and adhesion molecule expression, which are responsible for neutrophil recruitment [66]. Activated neutrophils release inflammatory mediators, angiogenic factors, lytic enzymes, and antimicrobial peptides [66] and play a major role in Th1 or Th17 recruitment through the production of CXCL9, CXCL10, or CCL20 [67, 68]. Neutrophil–lymphocyte interactions release survival factors that increase the lifespan of the short-lived neutrophils [19].

Chemokines and their receptors (CCL2/MCP-1, CCL5/RANTES, CXCL12/CXCR4) have an important impact on the development and maintenance of the cerebellum [69], and they are expressed in several parts of the brain including the cerebellum [70]. Moreover, chemokines may influence the cross talk between neuron and glial cell types and can function as a third communication system in the brain [71]. The cerebellum is a CNS structure whose development continues to occur in the postnatal period, leaving it susceptible to malformation events. The external granule cell layer (EGL) is formed during cerebellar development, when cerebellar granule cell progenitors produced in the upper rhombic lip (URL) migrate over the cerebellar primordium to form a secondary proliferative zone, the EGL. Formation of the internal granule cell layer (IGL) occurs during early postnatal development, when granule cell precursors in the outer zone of the EGL proliferate, migrate to the inner zone of the EGL, and exit from the cell cycle, differentiate, and radially migrate via the Purkinje cell layer to their final destination [72]. CXCL12 is a strong chemoattractant for granule cell progenitors in the URL, EGL, and dentate gyrus, also playing a critical role during neurogenesis through promotion of axonal growth and being expressed in embryonic and postnatal meninges that cover the cerebellum [73]. It is also known as a potent chemoattractant for URL cells, inhibiting CXCR4-expressing premature granule cell migration to the EGL [74]. Therefore, the irregular EGL formation could partially be attributed to defects in cell migration from URL to EGL. Focusing on a chemokine–receptor axis, CXCL12/CXCR4 could provide new therapeutic potential for cerebellum developmental disorders.

Astrocytes have various functions in the CNS, which support differentiation and homeostasis of neurons and influence synaptic activity. They are also responsible for the formation of the BBB [75]. The BBB constitutes an elaborate structure formed by specialized capillary endothelial cells, which, together with pericytes and perivascular glial cells, control exchanges between the CNS and the periphery. Intricate interactions between various cellular components in the BBB are crucial in establishing its function and maintaining the delicate homeostasis of the brain microenvironment [76]. Existence of numerous astrocytic end-feet near the BBB demonstrate their role in regulation of BBB permeability, which is increased by

humoral mediators that can be secreted by astrocytes as well as other glial cells, including endothelin-1, glutamate, IL-1β, IL-6, tumor necrosis factor (TNF), macrophage inflammatory protein (MIP)-2, and nitric oxide [77]. Astrocytes subsequently regulate neuronal differentiation and homeostasis, and evidence has shown that astrocytes interact with the immune system because they express a variety of PRRs, and both recognize danger signals and respond accordingly [78]. Following PRR activation, astrocytes produce cytokines, chemokines, and neurotrophins that target neighboring glial cells and neurons [78]. Therefore, the perception of immune privilege in the CNS can be minimized because astrocytes can reduce inflammation via releasing IL-27, and they also have constructive neuroprotective effects on the healthy brain [79]. Astrocyte activation leads to activation of damage control mechanisms such as induction of a neuroprotective effect and polarization toward Th2 profile. Conversely, IFN-γ produced by Th1 cells can suppress astrocytes that aggravate neuroinflammation [80]. Thus, astrocytes may constrain and defer neuroinflammation, but high levels of IFN-γ might promote astrocytes to become potent APCs and even promote inflammation [81].

The complement system includes nearly 40 soluble and membrane-bound proteins that play a critical role in host defense against pathogens and initiation of inflammation [82, 83]. The liver is the main source of complement production, but it can be produced by many types of resident cells in the CNS [84]. Complement receptor expression (C3a and C5a) has been shown on glial cells and neurons [85]. The complement system contributes to modulating CNS development and inflammation [86]. Complement components C1q and C3 are expressed on neurons throughout the CNS where they opsonize synapses to highlight them for phagocytosis by microglia [87]. Their expression peaks during crucial stages of neurodevelopment such as synapse formation and activity-dependent refinement [88]. MHC1 expression is also spread across the brain including cerebellar neurons and neuronal synaptic membranes, and MHC1 is thought to be fundamental for synapse formation and plasticity; potentially any defect in MHC1 could, thus, lead to cerebellar developmental disorders such as autism [89]. Systemic complement depletion diminishes perihematomal brain edema and TNF-α release following experimental intracerebral hemorrhage [90]. The core mechanism involving complement components in immune cells recruited into the brain and cerebellum parenchyma through the BBB remains unclear. Some therapeutic approaches using large recombinant molecules may work only when the BBB is compromised, while small molecule drugs, such as known receptor antagonists and low molecular weight heparin, could be potential therapeutics for treating patients with chronic disorders who have a non-compromised BBB. Blocking or preventing complement activation is a successful approach to decrease leukocyte recruitment and endothelial activation during CNS inflammation [78]. Therefore, specificity and balance challenges of various coincident cascades need to be highlighted; approaches that both promote beneficial effects and prevent detrimental activities are attractive goals for better understanding of human neurological disorders.

Adaptive Immune T and B Cells in the Cerebellum

Adaptive immunity is orchestrated by T-helper (Th) cell subsets, through secretion of lineage-specific cytokines. T cells enter the CNS and cerebellum parenchyma in several autoimmune, infectious, and degenerative neurological diseases. Therefore, T cells can be directly responsible for neuronal damage in many neurological diseases via different mechanisms of neuronal damage that are mediated through different T-cell subsets. For example, lesions of the vestibulocerebellum decrease the secretion of hematopoietic cytokines in the bone marrow and thymus tissue culture and decrease peripheral blood leukocyte concentration, neutrophil myeloperoxidase activity, and antibody response [91]. Conversely, the suppressive influence of vestibulocerebellar lesions on immune function demonstrate that induced lymphocyte proliferation is significantly enhanced on days 8, 16, and 32 following the effective kainic acid lesions in the bilateral cerebellar fastigial nuclei in rats [92]. Subsequently, cerebellar fastigial nuclei contribute to the modulation of lymphocyte function but not to the hypothalamic–pituitary–adrenal axis [92].

Although T cells within the CNS and cerebellum have been reported to be pathogenic cells, recent findings have demonstrated important functions for T cells in the healthy CNS [93]. Immunization of rats with copolymer (Cop-1), which mimics the myelin basic protein in the CNS and polarizes lymphocyte activation toward the Th2 profile, protects the injured optic nerve from secondary degeneration [94]. Moreover, regulatory T cells (Treg cells) reduce microglial activation after inflammation develops, and astrocytes promote Treg cell transcription factor expression [95]. Therefore, T cells are key players and might have a beneficial role in the development of CNS adaptive immunity.

The balance between Treg and inflammatory T cells (IFN-γ-producing Th1 and IL-17-producing Th17) is critical in neuroinflammatory diseases and contributes to the pathogenesis [96, 97]. Children with cerebellar developmental disorders such as autism displayed impaired immune profiles and function, which is characterized by a systemic deficit of $Foxp3^{+}$-(Treg) cells and increased expression of some transcription factors ($ROR\gamma t^{+}$, $T\text{-}bet^{+}$, $GATA\text{-}3^{+}$) [98]. This suggests the importance of transcription factor signaling, which results in an immunological imbalance in cerebellar developmental disorders. The balance between Treg cells and other T-cell subsets (Th1, Th2, Th17) seems to be important for cerebellum homeostasis, neurogenesis, and neuroinflammation. The immune system plays a crucial role in the recovery process of cerebellum development and disorders [99]. Researchers working on new therapeutic strategies have a cutting-edge understanding of the pathogenesis of many diseases and disorders, but there is no specific central therapy targeting Treg cells or suppressing Th1 or Th17 cells. It is currently unknown whether Treg cells can be selectively targeted. By better understanding the regulation of harmful effects compared with beneficial homeostasis promoting T-cell responses at the immune and central nervous systems, it is believed that novel potential therapeutic strategies will be identified, which could also avoid side effects of currently available immunosuppressive treatments.

Humoral immune responses controlled by B lymphocytes have been implicated in CNS and cerebellar diseases and disorders [99]. Recently, it was reported that the association of maternal autoimmune disorders with cerebellar developmental disorders in offspring may be regulated by the passive transfer to the fetus of maternal immunoglobulin G (IgG) antibodies that show reactivity to self-proteins in the mother or child [100]. Thus, pregnant women who have immune disorders or autoimmune reactions, even at a clinically undetectable level, may be linked with the production of maternal antibodies that can enter the fetal brain and potentially perturb fetal brain development. Collectively, immune responses are critical in cerebellum development, and balance of these responses is required to avoid cerebellar developmental diseases/disorders.

Purkinje cells are a class of GABAergic neurons located in the cerebellum that could shape adaptive immunity. Immunoglobulin plays a role in many neuro-disorders. Antibodies to cytoplasmic components of cerebellar Purkinje cells have frequently been labeled in serum and CSF [101]. However, the roles of such antibodies in the pathogenesis of neuronal injury are undefined. Intact neurons are thought to be essentially impermeable to IgG, and antibodies to cytoplasmic or nuclear neuronal antigens cannot enter neurons and bind to their intracellular targeted antigens [102]. The cerebellar Purkinje cell is a possible exception. Experimentally, Purkinje cells showed a high endocytic activity for a wide range of substances that originate from the ventricular CSF [103], and they can also incorporate IgG and S100 [101]. Therefore, the aptitude of Purkinje cells and related neurons to engulf antibodies is vital because of the possible role of autoantibodies in disease pathogenesis and because cerebellar injuries and Purkinje cell damage have been demonstrated in animals and human patients receiving IgG-conjugated immunotoxins [101].

Cerebellum and Immune Response Interactions in Cerebellar Diseases

Cerebellum and cerebellar Purkinje cells seem to be a common immunological target in some neurological disorders. This may be because the cerebellum is one of the largest, oldest, and most conserved structures in the nervous system and/or because Purkinje cells have good and various antigenic targets.

The immune system mediates the pathophysiology of cerebellar diseases via different immune responses. Evidence suggests that the cerebellum is a CNS target of autoimmunity, as shown by the high prevalence of paraneoplastic cerebellar degeneration (PCD) within paraneoplastic neurological syndromes [104]. Immune-mediated cerebellar ataxia, according to the associated autoantibodies, includes gluten ataxia, paraneoplastic cerebellar degeneration, anti-glutamic acid decarboxylase antibodies (GAD) antibody associated with cerebellar ataxia, and Hashimoto's encephalopathy (HE) [105]. Many of these autoantibodies distinguish cerebellar-specific antigens traced in the Purkinje cell soma to dendrites resulting in a Medusa-head immunohistochemical staining pattern [106]. There is a large amount of

evidence to suggest that the cerebellum can be a primary target for organ-specific autoimmune disease, and thus, the proposed term of primary autoimmune cerebellar ataxia (PACA) suggests that there is no known trigger factor for the development of immune-mediated damage to the cerebellum, but that it is more likely attributed to a hormonal imbalance, which impairs various immune responses such as in hypothyroidism, type 1 diabetes mellitus, and vitiligo. Therefore, humoral mechanisms, cell-mediated immunity, inflammation, and vascular injuries could contribute to the cerebellar discrepancies in immune-mediated cerebellar ataxia.

Some of the pathological damage to CNS is a result of immune-mediated mechanisms and not secondary to vitamin or nutrient deficiencies. Examination of patients with gluten ataxia revealed patchy loss of Purkinje cells in the cerebellar cortex [107]. Moreover, gluten ataxia is characterized by a diffuse infiltrate of T lymphocytes with a smaller number of B lymphocytes and macrophages in the cerebellar white matter and the posterior column of the spinal cord as well as loss of Purkinje cells [107]. Similar findings have been defined in patients with established celiac disease who then developed cerebellar ataxia [108]. Experimentally, antibody cross-reactivity between antigenic epitopes on Purkinje cells and gluten peptides has been reported [109]. Serum from patients with gluten ataxia and patients with celiac disease but with no neurological symptoms display cross-reactivity with epitopes on Purkinje cells using both human and rat cerebellum. The reactivity can be abolished after absorption of the antigliadin antibodies using crude gliadin. A study investigated the epitope responsible for cross-reaction between gliadin peptides and cerebellar peptides, by assessing the reactivity to specific peptides from the gliadin and cerebellum in serum from 50 autism patients and 50 healthy controls. Autism patients showed a significant increase in the antibodies against gliadin and the cerebellar peptides [110]. Therefore, this study suggests that a subgroup of patients with autism produce antibodies against Purkinje cells and gliadin peptides, which may be responsible for some of the neurological symptoms of autism. An antibody-mediated pathogenesis is also supported experimentally, revealing that intraventricular injection of serum from patients with gluten ataxia can induce ataxia in mice [111]. In conclusion, the brain–gut axis, the enteric nervous system, and the immune system contribute to the immune pathobiology of neurodevelopmental disorders through production of specific antibodies against cerebellum peptides to induce immune responses, which have detrimental effects on cerebellar tissues.

Communication between the gut and the brain, which is regarded as the gut–brain axis, is a well-known bidirectional neurohumoral communication system. Previous research that focused on the gut–brain axis mostly referred to its contribution in functional gastrointestinal syndromes, such as irritable bowel syndrome (IBS) [112]. It was recently reported that gut microbiota can modulate brain development and produce behavioral phenotypes via the gut–brain axis [113]. Thus, the potential effects of the microbiota–gut–brain axis in neurodevelopmental disorders are receiving much attention. The bidirectional communication in the microbiota–gut–brain axis acts mainly through both neuroendocrine and neuroimmune mechanisms. Moreover, the metabolites of microbiota can be absorbed and transported by the blood before crossing the BBB to modulate cerebral functions. The gut micro-

biota also contributes to cerebral developmental disorders by modulating the host immune response through releasing a storm of pro-inflammatory cytokines (including IL-1, IL-6, and IL-18) by intestinal epithelial cells, intestinal DCs, and macrophages [114]. Vagal afferents could be another potential mechanism by which the microbiota–gut–brain axis regulates communication, in which gut microbiota can send signals to the brain through the vagus nerve. Additionally, interruption of the microbiota–gut–brain axis in neurodevelopmental disorders such as autism is a comorbidity of neurodevelopmental deficits and intestinal symptoms. Moreover, autistic behaviors were often associated with gut microbiota dysbiosis [115]. Restoring the balance of the microbiota–gut–brain axis offers promising beneficial therapeutic effects on cerebellar developmental disorders such as autistic deficits.

Therefore, a link between the cerebellum and gastrointestinal tract might exist. Patients with gluten sensitivity and normal bowel mucosa (occasionally signified as potential celiac disease) have evidence of antibodies targeting tissue transglutaminase (TG) in the small bowel mucosa and at extraintestinal sites such as the CNS and/or cerebellum [116]. IgA deposition on the jejunal tissue transglutaminase has been reported in the jejunal tissue but also in the brain (mostly in cerebellum) of patients with gluten ataxia and in none of the controls [117]. This immune response described for gluten ataxia suggests a neural transglutaminase and results in clinical manifestations primarily in the brain or the peripheral nervous system, with minimal involvement of the gut; the gut may be involved through deposition of autoantibodies against brain transglutaminases (TG6) [107]. Thus, gluten ataxia is immune-mediated and belongs to the same spectrum of gluten sensitivity as celiac disease. Transglutaminases may play a critical role in pathogenesis of various signs seen in the context of gluten sensitivity. Thus, antibodies against TG6 may become novel markers for the neurological manifestations of gluten sensitivity. There is also cell-mediated immunopathogenesis. Most patients with celiac disease have HLA-DQ2 or HLA-DQ8 class II molecules that bind and present peptides derived from exogenous protein antigens to CD4 T cells. Thus, it has been hypothesized that T cells that react with gluten peptides play a major role in the pathophysiology of cerebellar ataxia because celiac disease is caused by an exogenous protein antigen and is linked to HLA-DQ2/HLA-DQ8 expression.

Conclusion

Understanding the links between the immune, CNS, enteric, and endocrine systems is fundamental to understand the bidirectional communication between the immune system and cerebellum. An imbalance in the neuroimmune interaction may promote the onset of autoimmune disorders and constitute an important component of pathogenic mechanisms involved in neurodevelopmental and neurodegenerative diseases such as autism and cerebellar ataxia (Fig. 1). The eventual challenge may be to elucidate how these various mechanisms of communication interact with each other.

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Teratogenic Influences on Cerebellar Development

Albert E. Chudley

Abstract The effects of environmental agents on cerebellar development are profound, and this organ has not been given the attention that is deserving of it, based on its importance in motor, cognitive, and behavioral functions. This chapter will review select agents associated with teratogenic effects on cerebellar structure and function. Mechanisms of teratogenesis and genetic influences will be addressed. The emerging role of effects of environmental agents and effect on the epigenetic mechanisms and gene expression are discussed. Prenatal alcohol exposure and fetal alcohol spectrum disorder will be discussed in greater detail, as this disorder is the most common teratogenic disorder affecting humans. Indeed, many of the phenotypic effects of FASD are the result of cerebellar injury and dysfunction.

Keywords Teratogenesis • Brain imaging • Birth defects • Prenatal exposures • Viral infections • Zika virus • Rubella • Anticonvulsants • Valproic acid • Alcohol • Genetic factors • Epigenetics • Fetal alcohol spectrum disorder

Introduction

Teratology can be defined as science dealing with causes, mechanisms, and manifestations of developmental deviations of either structural or functional nature [1, 2]. A teratogen is any agent that compromises a healthy intrauterine environment and results in altering normal development during the period of embryonic or fetal development resulting in abnormal structure or function, restriction of growth, or death of the embryo or fetus [3]. Known teratogenic agents include infectious agents (e.g., rubella virus, Zika virus, cytomegalovirus, toxoplasmosis, varicella, etc.), a chemical or drug (most anticonvulsant medications such as phenobarbital, diphenylhydantoin, valproic acid, retinoic acid, warfarin, etc.), heavy metals and

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environmental poisons (mercury, lead, manganese, and toluene/benzene derivatives), excessive radiation, maternal conditions (drug and alcohol abuse or addiction to illicit drugs, smoking, nutritional deficiencies, metabolic disorders in the mother such as phenylketonuria, diabetes, mental and emotional stress, etc.), invasive medical interventions (such as amniocentesis, chorionic villus sampling, etc.), and changes in the environment (elevated core temperature for an extended period of time such as febrile illness, sauna or hot tub use, etc.) [4–6].

Teratogens in humans have certain characteristics that include evidence of an increase in the frequency of a known abnormal phenotypic effect, such as neurobehavioral changes or structural changes leading to birth defects, a dose response relationship with a threshold effect, critical periods of significant risk, established mechanism of action, biological plausibility of teratogenicity, and genetic and/or epigenetic predisposing risk factors. Identifying and confirming the etiological origins of birth defects can lead to better treatment and prevention and, in the case of infectious diseases, the development of effective vaccines to reduce the risk in the population [2].

The effects of teratogens are variable and dependent on timing of the exposure, the dose of the exposure, the frequency of exposure(s), maternal and fetal genetic factors, and other mitigating or susceptibility factors that modify the effect. The exposure can lead to a variety of outcomes, from apparently normal and unaffected to mild impairment and to severe impairments with multiple malformations, or result in abortion and death.

As with all developing organs, the brain is often the target of teratogenic effects. The resulting impairments from a teratogenic exposure affecting brain development can lead to effects on brain structure (cellular defects, malformations, or disruption) and/or brain function that can manifest as behavioral abnormalities, craniofacial dysmorphology, developmental delays, intellectual impairment, and/or severe physical disability. It is rare for a teratogenic effect to be restricted to a single organ structure or specific region of the brain. However, for the purposes of this chapter, emphasis will be placed on the teratogenic effect on the cerebellum and the clinical consequences.

The cerebellum is relatively small, but it has established functional connections to many other regions of the brain. Prenatal and postnatal injuries due to a variety of toxins result in neurologic deficits, including ataxia, hypotonia, dysarthria, and ocular motility problems. These exposures can present with impairments in movement, motor coordination, sensory function, cognition, affect regulation, or mood. Dysfunction of the cerebellum and its effects on connectivity to other brain regions have been correlated with a number of neurodevelopmental disorders that include autism, attention deficit hyperactivity disorder, dyslexia, as well as psychiatric diseases such as schizophrenia and bipolar diseases [7]. Many inherited disorders involving abnormal development and function of the cerebellum including cerebellar hypoplasia have been described [8].

The nature of the injury or exposure would be dependent on the subregions of the cerebellum involved and determined by alterations in the corresponding cerebro-

cerebellar circuitry [9]. Recent studies exploring the role of speech and language have demonstrated an important role of the cerebellum in communication in health and disease. Mariën et al. [10], in a consensus review of this topic, summarized their findings to date "cerebellar involvement in language extends far beyond the pure motor domain to a variety of high-level non-motor linguistic processes at both the expressive and receptive language level. In general the role of the cerebellum in language adds evidence to the view that timing and sequencing processing, sensorimotor adaptation and cognitive skill automatization act as the overall operational modes of the cognitive cerebellum."

Developmental abnormalities of the cerebellum have been induced by several teratogenic agents, including therapeutic agents such as 13-cis retinoic acid (Accutane©) and misoprostol (Cytotec©)l. [11–13]. Many early studies, prior to the 1970s, were limited in describing cerebellar abnormalities since techniques to visualize this organ were crude or not yet available for wide clinical use. Evaluation of the brain in the 1960s and 1970s was restricted to investigations such as electroencephalograms (EEG), pneumoencephalograms, ultrasound, and the earlier generation computed tomography (CT) or autopsy findings. The list of disorders with identifiable cerebellar lesions is growing particularly with the advent and ubiquitous use of newer imaging techniques. With the advent of newer imaging modalities, brain imaging has been enhanced. Single-photon emission computed tomography (SPECT) can provide 3D information, and positron emission tomography (PET) can help assess functional abnormalities in the brain before anatomical changes occur in many diseases of the brain. Using magnetic resonance imaging (MRI), structural CNS defects and malformations are more readily and accurately defined, or in the case of functional MRI analysis, brain activation responses to a variety of external stimuli can be visualized. Magnetic resonance spectroscopy (MRS) can identify disturbances in the neurochemistry of the brain. Diffusion tensor imaging (DTI) assesses the integrity of the white matter and maps normal and aberrant white matter tracts and brain circuitry. In this chapter some examples of teratogenic agents with effects on the developing cerebellum will be presented.

Intrauterine Infections

There are scores of infectious agents associated with intrauterine viral and parasitic infections. Most can cause a variety of developmental defects in exposed fetuses. Examples include the classical group of teratogenic pathogens, the so-called "TORCH" (*Toxoplasma gondii*, *O*thers like *Treponema pallidum*, *R*ubella virus, *C*ytomegalovirus, *H*erpes simplex virus), and other agents including Parvovirus B19, *Varicella zoster* virus, and *plasmodium falciparum* to name a few. In this chapter reviews of Rubella and the Zika virus are presented for illustration purposes, and readers are referred to recent reviews on intrauterine infections for further information [14, 15].

Congenital Rubella

As noted, several infectious agents have been implicated in causing birth defects and brain abnormalities [16]. The first report of a teratogenic agent in humans was made in 1941 by an Australian ophthalmologist Normal Gregg, who described children with cataracts as a result of rubella in the children's mothers during the pregnancy [17]. Congenital rubella is typically associated with other CNS abnormalities, microcephaly, growth retardation, congenital hepatitis, deafness, cataracts, retinopathy, and cardiovascular defects. The mechanisms of teratogenesis have included inhibited cell growth, impaired blood flow, direct effects of the ongoing infection with cytopathic effects, and immunopathological mechanisms [18, 19].

Townsend et al. [20] reported on a case of progressive panencephalitis in a child who was born with congenital rubella. Neuropathologic studies showed findings in the brain which included diffuse destruction of white matter with perivascular inflammatory cells and gliosis, moderate neuronal loss, numerous amorphous vascular deposits in the white matter, and severe generalized cerebellar atrophy. Recently Cluver et al. [21] reported on an infant with confirmed early prenatal rubella infection born with agenesis of the inferior cerebellar vermis. The authors suggest that the cerebellar defect was likely the result of spread of the virus through the vascular system causing vasculitis and endothelial necrosis [22]. There are only rare reports of cerebellar defects in congenital rubella syndrome.

It is likely that most viral and other infectious agents causing intrauterine infections have similar mechanisms of teratogenesis affecting the developing cerebellum [16, 23, 24]. Further investigations could clarify the role of viral infections in overstimulation of excitatory amino acid receptors, excess production of angiogenesis, pro-inflammatory cytokine neurotrophic factors, and apoptotic-inducing factors [25].

Congenital Zika Infection

Recently, the *Aedes* species mosquito-borne Zika virus has been confirmed to be causative of congenital microcephaly and other birth defects including arthrogryposis and sensorineural hearing loss [26–32]. The Zika virus belongs to a family of related arthropod-borne (arbovirus) that includes dengue, yellow fever, West Nile encephalitis, and Japanese encephalitis viruses and another virus from a different family, Chikungunya virus [30]. The virus was first recognized in the Zika forest of Uganda from a rhesus monkey with an acute febrile illness in 1947 [33] with human infections first reported in Nigeria in 1954 [34]. Subsequent spread to the Yap Islands of Micronesia, the Pacific Islands, and Polynesia showed that this was not a benign disease in humans [30]. From mid-2015 to 2016, over 30,000 cases were reported in Brazil [29] and subsequently as far north as Florida [35]. Several cases have been imported to European countries and North America including Canada

[36]. In a series of 23 infants from Brazil, de Fatima et al. [27] and Hazin et al. [37] identified common findings in the brain of these children through CT and MRI techniques. The abnormalities included brain calcifications in the junction between cortical and subcortical white matter; malformations of cortical development with simplified gyral patterns, pachygyria, or polymicrogyria in the frontal lobes; enlarged cisterna magna; abnormalities of corpus callosum; ventriculomegaly; delayed myelinization; and hypoplasia of the cerebellum and brainstem [37]. Garcez et al. [38] experimental studies on human brain culture confirm that the Zika virus abrogates neurogenesis during human brain development. Tang et al. [39] showed that there is a downregulation of genes involved in cell-cycle pathways, dysregulation of cell proliferation, and upregulation of genes involved in apoptotic pathways resulting in cell death. Clearly until an effective vaccine is developed [40], better treatment and diagnostic capabilities need to be developed and priority given to vector control. Outcomes of children born with congenital Zika virus infection show major CNS abnormalities and have features of severe delays in development and severe neurological dysfunction [27, 41].

Congenital Anticonvulsant Syndrome

It is estimated that well over a million women of childbearing age in the United States have epilepsy, the vast majority of whom are on drug therapy for management of this common disorder [42]. This is a concern since almost all antiepileptic drugs have potential risks for causing fetal anomalies and later developmental delay. This was first confirmed a reality in the early 1970s and 1980s with reports of children born to epileptic mothers on drugs that included phenobarbital, phenytoin, and carbamazepine presenting with recurrent patterns of birth defects that included major malformations, such as microcephaly, growth retardation, and minor craniofacial and digital/limb anomalies [43–50] (Fig. 1). Holmes et al. [50] showed that the risk of malformations was higher in women taking one anticonvulsants over women delivering babies who were on no anticonvulsants (odds ratio 2.8), and the risk when women were taking two or more anticonvulsants was even higher (odds ratio 4.2). Women with epilepsy who were not on medication in the pregnancy showed no increase in major congenital anomalies than the controls. Morrow et al. [51] studied pregnant women with a diagnosis of epilepsy in UK centers using a prospective, observational, registration, and follow-up approach. They found 4.2% of women delivered infants with major congenital malformations with a history of taking anticonvulsant medication. For polytherapy use, the rate was 6.0%, for monotherapy it was 3.7%, and for women with epilepsy taking no medication, the rate was 3.5%. Valproic acid demonstrated the highest rate of major congenital malformations at 6.2%. This is compared with the expected "background" rate of major congenital malformations as between 1% and 2% in the general population at birth [52, 53]. It has been suggested that some of the difference may be due to genetic factors that increase the frequency of anomalies in some children. This seems to be

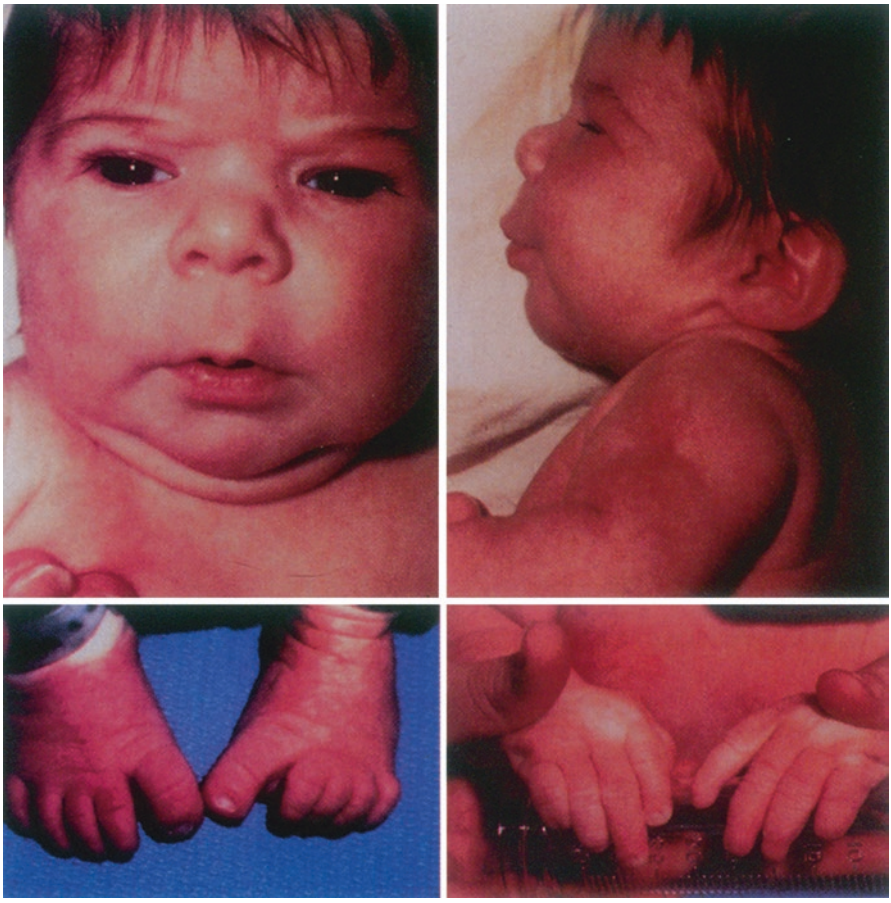

Fig. 1 Infant with typical facial features and distal digital hypoplasia with fetal hydantoin syndrome (From Buehler et al. *NEJM*. 1998. With permission)

borne out by studies that show differences in activity of the detoxifying enzyme epoxide hydrolase, with deficiency of the enzyme in infants, presenting with clinical features of hydantoin embryopathy [54, 55]. It has been hypothesized that anticonvulsants increase the production of free radicals resulting in vulnerability to malformations as a potential etiological factor [56].

There are several anticonvulsants in common use today. The list of anticonvulsants is long, and the most commonly used drugs include valproic acid, phenobarbital, phenytoin, carbamazepine, gabapentin, lamotrigine, levetiracetam, topiramate, vigabatrin, and benzodiazepines. A detailed review of the effects of valproic acid on human development including the cerebellum is presented below.

Valproic Acid

Valproic acid (VPA) is a widely used and effective anticonvulsant medication that is also used in the treatment of mood disorders, schizophrenia, and migraine headaches. Animal and human studies show that VPA is associated with a predictably higher rate of major congenital malformations that is dose dependent [57]. The risk is two to three times that of the expected rates of malformations in the population, and it is associated with a higher risk than are other anticonvulsants.

The risk of adverse outcomes following the use of VPA includes major congenital malformation including spina bifida, atrial septal defects of the heart, craniosynostosis, cleft palate, hypospadias, and polydactyly [53]. In 1984, DiLiberti et al. [58] described a consistent constellation of dysmorphic features that they called fetal valproate syndrome which has been confirmed subsequently in many reports [59, 60]. Although periconceptional use of folic acid is recommended for all women, those using anticonvulsants may benefit by using a higher dose of this vitamin, although evidence suggests that folic acid may not be protective in preventing spina bifida from occurring after exposure to VPA. This then begs the question what is the mechanism of the malformations in VPA and other anticonvulsants [44, 61]. VPA is also associated with neurodevelopmental and cognitive impairments [62] and is a known risk for autism spectrum disorders [63–65]. Christiansen et al. [64] confirmed in their prospective study that maternal use of VPA was associated with a significantly increased risk of autism spectrum disorder even after adjusting for maternal epilepsy. It is of interest and perhaps not coincidental that one of the effects of prenatal exposure to VPA is an increased risk for autism as well as cerebellar anomalies. A subgroup of children with autism and a subgroup of children exposed to VPA both demonstrate structural cerebellar anomalies. The most common model used in environmentally induced ASD models in rodents is the one induced by VPA [66].

Not infrequent and severe consequences of long-term postnatal use of phenytoin and VPA include cerebellar atrophy [67–70]. Although the mechanism of both prenatal and acquired postnatal effects on the cerebellum may be different, genetic studies suggest that the risk of cerebellar complications may be determined by variations in enzyme activities that metabolize drugs. Buehler et al. [54] showed this to be a fact. They studied infants with fetal hydantoin syndrome and confirmed reduced activity of epoxide hydrolase in those exposed and affected compared to both those exposed and unaffected and normal controls. CYP2C9 mutation (*2 or *3) reduces phenytoin metabolism by 25–50% and can increase the risk of phenytoin-related side effects. CYP2C9 polymorphism has been associated with a reduction in cerebellar white matter volume in epileptic users of phenytoin [69]. Animal studies confirmed that prenatal exposure to VPA is associated with loss of volume in the vermis and hemispheres. Ingram et al. [64] identified reduced Purkinje cells in the vermis with greater loss in the posterior lobe with parallel in some human autistic populations.

As newer and safer drugs become available for the treatment of epilepsy and other seizure disorders in women of childbearing age, the use of drugs such as VPA will likely continue to be reduced. It is important that women on these drugs need to be advised of the risks in pregnancy and screening measures and ongoing surveillance to assess fetal well-being be instituted.

Prenatal Alcohol Effects and Fetal Alcohol Sepctrum Disorder

Whether prenatal alcohol exposure (PAE) can harm the human embryo and fetus has been a contentious issue over the past century. Following seminal studies by Lemoine et al. [71] in France in 1968 and Jones et al. [72, 73] in the United States in 1973 did the irrefutable evidence of the harmful effects of alcohol in pregnancy become clear, and PAE is considered the most common teratogenic agent in humans. Based on extensive research in animals and humans, PAE has been demonstrated to cause a variety of structural and/or functional deficits in the developing fetus, even after a single binge episode or equivalent use in experimental situations [74–76].

In humans, the first reports were on infants and young children born to mothers who were known alcoholics. These children typically presented with intrauterine growth retardation, microcephaly, characteristic facial dysmorphic features of short palpebral fissure lengths of the eyes, abnormal and short midface with a smooth poorly formed philtrum and a thin vermilion border of the upper lip, risk to various birth defects including cleft palate, cardiac malformations, limb anomalies, and an increase in minor anomalies, with cognitive impairment and behavioral problems (Fig. 2). This presentation was called fetal alcohol syndrome (FAS) [73, 74, 77, 78]. Subsequently less visible signs of the prenatal effects of alcohol were identified in which affected children showed few or little of the facial and growth features but presented with cognitive and behavioral difficulties. The use of other terminologies such as fetal alcohol effects (FAE), partial fetal alcohol syndrome (pFAS), and alcohol-related neurodevelopmental disorder (ARND) was applied

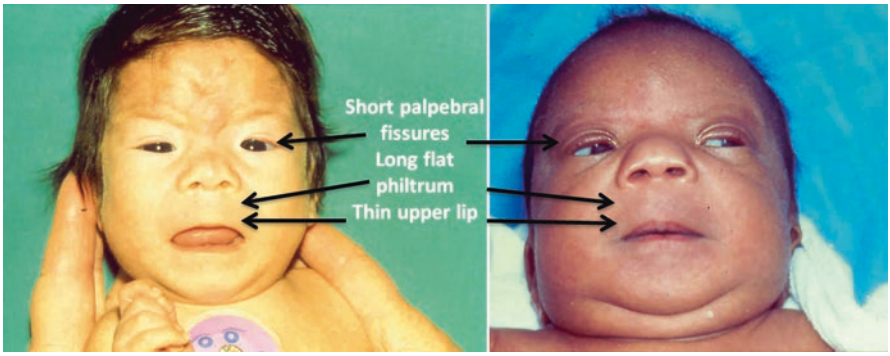

Fig. 2 The typical facial features of fetal alcohol syndrome in two infants

[79–85]. The term fetal alcohol spectrum disorder has often been used to include the whole spectrum of effects of PAE. Cook et al. [84] recently updated the fetal alcohol spectrum disorder (FASD) diagnostic guidelines in Canada, and the terminology has been changed to include two diagnostic categories, FASD with sentinel facial features (FAS) and FASD without sentinel facial features (previously called partial FAS and ARND).

The diagnosis of FASD requires multidisciplinary team assessments to identify behavioral, cognitive, neurological, and dysmorphic features congruent with FASD [82]. This means that referrals for suspected cases are sent to the multidisciplinary team for a thorough evaluation by other specialists that includes specialist physicians (developmental pediatricians, geneticists, psychologists, speech and language therapists, occupational therapists, education specialists, and social work case workers). Details of the referral process, evaluations and steps in the diagnosis, and management recommendations are described in detail elsewhere [82, 84].

Evaluation of the brain is an important component of diagnosis. This includes an in-depth assessment of brain function using standardized testing of (1) cognition, (2) memory, (3) language, (4) academic achievement, (5) executive function (including impulse control and hyperactivity, adaptive behavior, social behavior, social skills or social communication, attention, affect regulation, motor skills, and neurological assessment of brain size, neuroanatomy, and neurophysiology (including neurologic examination and in some cases imaging)) [84].

There are many other conditions that can mimic FASD with an extensive differential diagnosis [86], and many comorbid conditions are often co-occurring in FASD individuals, some conditions at rates greater than 100 times the general population based on US data [87]. These children need to be identified as early as possible if therapy and interventions are to make a difference in their long-term prognosis, and so screening programs need to be introduced to afford early detection [88]. Many affected children and adults who are not identified or diagnosed until later in life can experience what has been referred to as secondary disabilities [89]. They can be lost in society and can experience apprehension by social service agents and foster care, school failure with early dropout, addiction problems, mental health difficulties, limited employment opportunities, homelessness, and involvement with crime and the justice system with frequent incarceration [89, 90].

The prevalence of fetal alcohol spectrum disorder (FASD) is estimated to be between 2.4% and 4.8% in a school-age population in the United States [91] and similar high rates of prevalence in a school-age population in Italy [92]. The highest rates at 18–26% were estimated in an at risk rural and lower socioeconomic community in South Africa [93]. Because of the high prevalence in most populations studied and the high costs to society of the condition, prevention of drinking in pregnancy should be a high priority of governments, social and health care professionals, and the alcohol industry [87, 94–99].

It is relevant that several of the brain domain impairments observed in PAE and FASD individuals exhibit these difficulties, in part, because of teratogenic effects of alcohol on the cerebellum and their respective connections to other regions of the brain. For example, the functions of motor and balance, eye tracking and visual–

spacial perception, cognitive abilities, learning, language, emotional responses, and attention pathways are connected to the cerebellum. Many children with FASD have impairments in these functions. Many research reports and clinical descriptions in the literature to support the above association of cerebellar dysfunction and FASD are presented in the following pages.

Mechanisms for Alcohol Teratogenesis

Ethanol is toxic to the developing embryo and fetus. Alcohol readily crosses the placenta and the blood–brain barrier. Alcohol can affect normal placental function and cause altered blood flow, ischemia, and hypoxia to the fetus. There is also an interaction between the direct toxic effects and indirect or maternally mediated effects of alcohol [100].The mechanisms are complex and involve variables in the timing, frequency, and dose of exposure. Alcohol is known to act on or modulate many different target molecules' multiple mechanisms, activated at different stages of embryonic and fetal development or at different dose thresholds of exposure and stages of development, resulting in diverse phenotypes [101–103]. The earlier the exposure of teratogenic factors during organogenesis, the greater the harm that is likely to occur [74, 103–105].

Molecular Pathways and Genetic Factors

PAE and FASD are perhaps best considered to be a prototypical multifactorial teratogenic disorder whereby both genetic predisposing factors and environmental exposures combine to have a variable phenotype (Fig. 3). It is evident that alcohol alone can be directly toxic to the embryo and fetus, but other factors also can either contribute to the risk (as aggravating factors) or have protective effects to some degree (a mitigating factor). PAE is both dose dependent (acute vs chronic exposure; frequency of exposure) and sensitive to critical periods of developmental stage. Factors shown to be protective include good nutrition prenatally and after

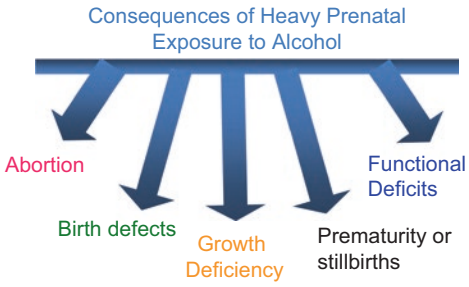

Fig. 3 Variable fetal outcomes from excessive ethanol exposure

birth [106], consistent and nurturing child care, early diagnosis with earlier interventions, and favorable genetic factors (particularly those involved in alcohol metabolism). According to May and Gossage [107], maternal risk is multidimensional, including factors related to quantity, frequency, and timing of alcohol exposure, maternal age, number of pregnancies, number of times the mother has given birth, the mother's body size, nutrition, socioeconomic status, metabolism, religion, spirituality, depression, other drug use, and social relationships. Some risk factors in the child include poor nutrition; exposure to neglect; physical, emotional, or sexual abuse; repeated changes in caregivers and place of residence; "unfavorable" genetics; and a diagnosis later in childhood [89]. It is well established that the genetic background of the mother and fetus influences the risk of ethanol-induced malformations [108]. The more efficient alcohol dehydrogenase (ADH) allele, ADH 1B*3, affords protection for FASD outcomes [109], while the maternal and fetal ADH1B*2 allele reduced the risk for FAS in a South African population (in comparison with ADH1B*1) [108]. For more recent reviews relevant to the importance of polymorphisms in the alcohol metabolizing pathway, the reader is referred to other reviews [110, 111] (Figs. 4 and 5).

A recent population-based prospective children's health and development study from Britain confirmed a genetic risk to some children genetically predisposed to the effects of alcohol exposure in pregnancy [112]. The authors found four ADH genetic variants in alcohol metabolizing genes in 4,167 children which were strongly

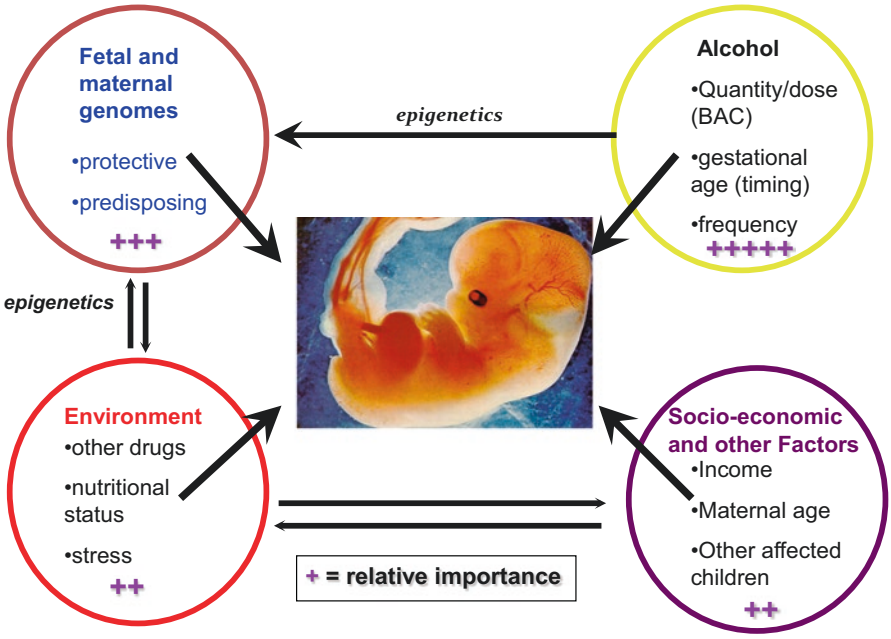

Fig. 4 A schematic representation of risk factors contributing to FASD

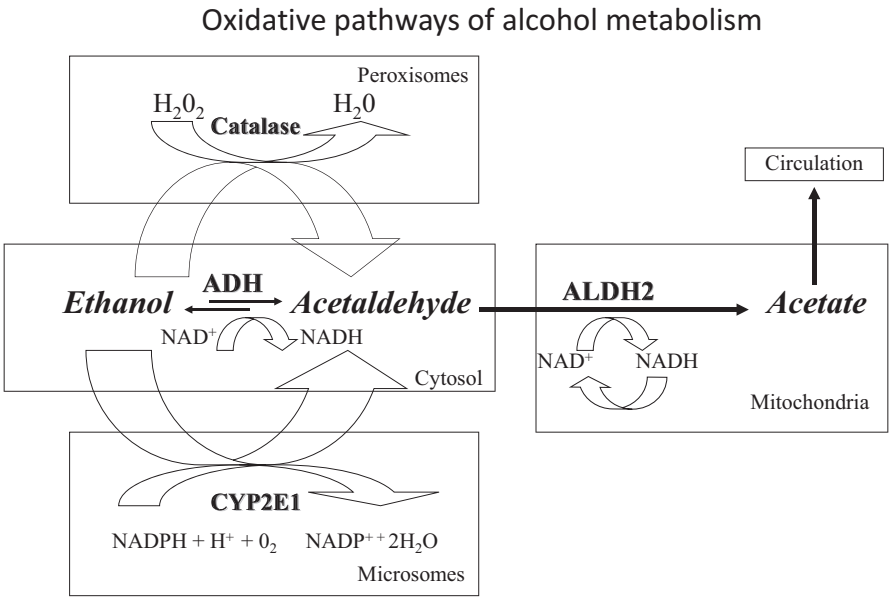

Fig. 5 Oxidative pathways of alcohol metabolism. The enzymes alcohol dehydrogenase (*ADH*), cytochrome P450 2E1 (*CYP2E1*), and catalase all contribute to oxidative metabolism of alcohol. *ADH*, present in the fluid of the cell (i.e., cytosol), converts alcohol (i.e., ethanol) to acetaldehyde. This reaction involves an intermediate carrier of electrons, nicotinamide adenine dinucleotide (*NAD+*), which is reduced by two electrons to form *NADH*. Catalase, located in cell bodies called peroxisomes, requires hydrogen peroxide (*H2O2*) to oxidize alcohol. *CYP2E1*, present predominantly in the cell's microsomes, assumes an important role in metabolizing ethanol to acetaldehyde at elevated ethanol concentrations. Acetaldehyde is metabolized mainly by aldehyde dehydrogenase 2 (*ALDH2*) in the mitochondria to form acetate and *NADH* (From Chudley [187])

related to lower IQ at age 8, as was a risk allele score based on these four variants. All the mothers of these children took moderate amounts of alcohol during the pregnancy. The authors suggest that, even among women drinking moderate amounts of alcohol, subtle changes in exposure to alcohol due to an ability to metabolize the substrate may be important and offer some support to the hypothesis that even small amounts of alcohol in utero have an effect on future cognitive outcomes.

Alterations in a number of molecular pathways have been suggested as candidates responsible for the range of FASD phenotypes [101, 113, 114]. These include (1) alterations in the regulation of gene expression (e.g., reduced retinoic acid signaling [115, 116], homeobox gene expression, altered DNA methylation [117]); (2) interference with mitogenic and growth factor responses involved in neural stem cell proliferation, migration, and differentiation [118]; (3) disturbances in molecules that mediate cell–cell interactions (L1, NCAM, loss of trophic support, e.g., [119, 120]); (4) activation of molecular signaling controlling cell survival or death (growth factor deprivation, oxidative stress, apoptotic signaling and caspase-3 activation, suppression of NMDA glutamate and GABAA receptors, withdrawal-induced

glutamatergic excitotoxicity), [121, 122]; (5) and derangements in glial proliferation, differentiation, and functioning [123].

Lombard et al. [124] utilized a computational candidate gene selection method that identified genes that may play a role in alcohol teratogenesis. Using a modification of the methodology called convergent functional genomics which combines data from human and animal studies, this group identified a short list of high-probability candidate genes, with the inclusion of additional lines of evidence in the presence of limited expression studies in an animal model and the absence of FAS linkage studies. From a list of 87 genes, the group prioritized key biological pathways significantly overrepresented among the top-ranked candidate genes. These pathways include the TGF-β signaling pathway, MAPK signaling pathway, and the Hedgehog signaling pathway.

The genes in the TGF-β signaling pathway may play pivotal roles during embryogenesis and development and have a potential role in the distinct characteristics associated with FAS, i.e., CNS dysfunction, craniofacial abnormalities, and growth retardation. CNS dysfunction is the most severe and permanent consequence of in utero alcohol exposure and the only feature present in all diagnostic categories in FASD. These observations make the TGF-β signaling pathway an important consideration, as it is essential in fetal and CNS development. Alcohol inhibits TGF-β-regulated processes such as cortical cell proliferation and neuronal migration, disrupts axonal (the major extension of a nerve cell) growth, and upregulates cell adhesion molecule expression [125]. TGF-β signaling pathway interacts with alcohol, and/or its metabolic breakdown products, and alcohol may have a detrimental effect on the efficiency of this developmentally essential pathway.

The MAPK pathway transmits many signals, leading to growth, differentiation, inflammation, and apoptosis responses [126]. This pathway is very complex and includes many protein components. MAPK pathway components are involved in the regulation of meiosis, mitosis, and postmitotic functions and in cell differentiation. The MAPK signaling pathway can be activated by a variety of stimuli as well as external stress factors, such as alcohol [127]. Using a mouse model of FAS, experimental manipulation of second-messenger pathways (that also impact on the MAPK pathway) completely reversed the action of ethanol on neuronal migration in vitro as well as in vivo [128].

The Hedgehog signaling pathway was also identified to contain several genes within the candidate list. This signaling pathway is a highly conserved and key regulator of embryonic development. Knockout mouse models lacking components of this pathway have been observed to develop malformations in the CNS, musculoskeletal system, gastrointestinal tract, and lungs [129]. FAS animal models have a similar craniofacial phenotype to mouse models treated with antibodies that block Hedgehog signaling components, specifically the sonic Hedgehog (Shh) molecule [130, 131 132]. Alcohol resulted in a significant decrease in Shh levels in the developing embryo, as well as a decrease in the level of other transcripts involved in Shh signaling. Addition of Shh after alcohol exposure led to fewer apoptotic (dead or dying) cranial neural crest cells and a decrease in craniofacial anomalies [131]. Altered function of genes in the Hedgehog signaling pathway may thus contribute to the brain malformations and dysfunction in FASD.

Epigenetics

Epigenetic mechanism as a cause of the diverse effect of PAE and FASD is emerging as a potentially important mediator of the FASD phenotype [133–136]. Epigenetics refers to modifications of DNA and its packaging that alter the accessibility of DNA to potentially regulate gene expression and cellular function without changes to the underlying genomic sequences [135, 137]. There are several mechanisms in which gene expression can be controlled, and the most studied epigenetic modification in human populations is DNA methylation. DNA methylation generally represses gene expression, but this relationship is less well defined for CpGs located within gene bodies and intergenic regions [138]. Furthermore, DNA methylation is closely associated with several key developmental processes, including genomic imprinting, tissue specification, and differentiation [139]. Prenatal alcohol exposure has been shown in animal studies to alter methylation which is predicted to alter gene expression and thus alter developmental processes [134, 140, 141].

There have been few human studies to test the role of changes in methylation and relationship to FASD. Several studies have demonstrated the effect of PAE on the *H19* imprinted gene in both mice and humans [142, 143]. Altered expression of the *H19* gene could interfere with normal growth mediated through the *Igf2* gene. A smaller human study characterized the DNA methylation profile in buccal epithelial cells (BECs) from a small cohort of human FASD samples, identifying alterations in the epigenome of children with FASD, particularly within the protocadherin gene clusters, which are involved in producing proteins involved in cell adhesion [144]. A genome-wide DNA methylation study in mouse embryos exposed to ethanol also identified significant changes within several imprinted genes including both *H19* and *SLC22A18* [145]). The *SLC22A18* gene is located in an imprinted region and plays a role in tumor suppression with other genes in the region mediating growth. A recent comparatively large study compared a cohort of FASD and alcohol-exposed children with controls through genome-wide DNA methylation patterns of BECs which were analyzed (Portales). Results from the study by Portales-Casamar et al. [146] further confirmed these findings, as five down-methylated probes in *H19* and six in *SLC22A18* were altered in the FASD cohort. With validation, these findings provide initial insight into the molecular mechanisms underlying the effects of PAE on children and present a potential role for DNA methylation in the etiology of FASD. It may also be possible to define a biomarker for alcohol exposure that may aid in the earlier diagnosis, referral, and treatment of this common disorder.

FASD and the Cerebellum

The earliest autopsy studies described in humans diagnosed with FAS and PAE identified errors in cell migration, agenesis or thinning of the corpus callosum, and anomalies in the cerebellum and brain stem [73, 147–149]. Subsequent imaging

studies with newer technology and resolution were consistent with autopsy findings [150]. These showed overall volume reductions in the cranial, cerebral, and cerebellar vaults in FASD [151–156]. Furthermore, other studies have suggested that this decrease is not uniform but rather that the parietal lobe [153–155, 157], portions of the frontal lobe [154], and specific areas of the cerebellum [156, 158, 159] appear to be especially sensitive to alcohol insult (Fig. 6).

Studies of effects on brain volume using imaging techniques have reported disproportionate size reductions in the cerebellum [153, 156, 160–162]. Cardenas et al. [162] studied PAE individuals using a cerebellar parcellation tool kit with T1-weighted MRI to assess cerebellar size. They concluded that (1) PAE-related microcephaly is strongly related to cerebellar hemispheric volumes and (2) smaller cerebellar measures in FASD are not fully explained by microcephaly and suggest an additional direct effect of prenatal alcohol exposure on the cerebellum.

Experimental studies on animals confirmed that PAE targets certain areas of the brain, particularly the cerebellum and the craniofacial structures [74, 163, 164]. Nathaniel et al. [165, 166] showed that the cerebellum and the area and circumference of the vermal cerebellum were significantly reduced in ethanol-exposed pups compared with the pair-fed controls. Studies in rats showed that synaptic density of the molecular layer of the cerebellar lobule VI was decreased in 28-day-old animals which were exposed prenatally to ethanol [167].

Studies in the mouse cerebellum showed that microglia promote the death and subsequent engulfment of Purkinje cells that express activated caspase-3 when they are undergoing synaptogenesis [168]. Similar results were observed in a developing nematode *C. elegans*, where cells in the advanced caspase (CED-3)-dependent stage of degeneration could recover [169]. Sawant et al. [170] assessed fetal cerebellar

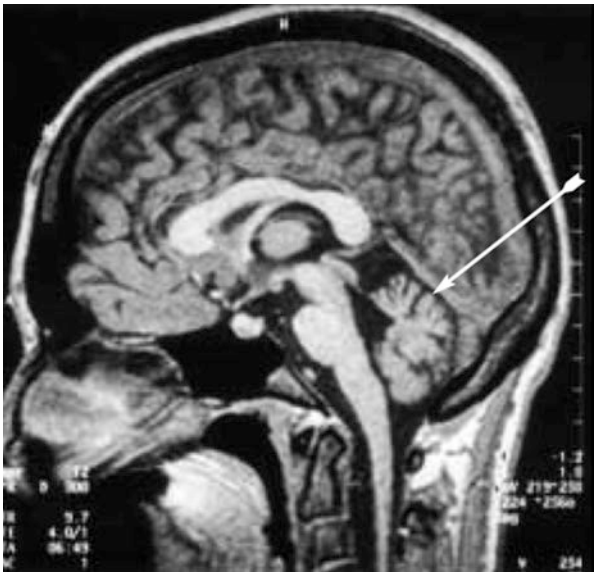

Fig. 6 A MRI demonstrating a small cerebellum and vermis hypoplasia (*arrow*) in a child with FAS (From Fig. 1 in Autti-Rämö et al. [156].)

Purkinje cell counts in an early-maturing region (lobules I–X) and a late-maturing region (lobules VIc–VII) from midsagittal sections of the cerebellar vermis in sheep. Third trimester-equivalent ethanol exposure caused a significant reduction in the fetal cerebellar Purkinje cell volume density and Purkinje cell number only in the early-maturing region, and as expected, the first trimester-equivalent ethanol exposure resulted in significant reductions in both the early- and late-maturing regions. The authors concluded prenatal ethanol exposure in the first trimester interferes with the genesis of Purkinje cells in an unselective manner, whereas exposure during the third trimester selectively kills postmitotic Purkinje cells in specific vermal regions during a vulnerable period of differentiation and synaptogenesis.

Chronic prenatal alcohol exposure on the immature central nervous system (CNS) profoundly inhibits insulin and insulin-like growth factor (IGF) signaling [171, 172]. They conclude that insulin-stimulated central nervous system neuronal survival mechanisms are significantly impaired by chronic gestational exposure to ethanol and that the abnormalities in insulin signaling mechanisms persist in the early postnatal period, which is critical for brain development. The same research group [173] observed ethanol dose-dependent reductions in cerebellar aspartyl (asparaginyl)-β-hydroxylase (AAH) immunoreactivity and significant impairments in insulin- and IGF-I-stimulated directional motility in granule neurons isolated from ethanol-exposed rat pup cerebella. In addition to reduced motility, the authors observed that chronic in vivo ethanol exposure mainly reduced the percentages of migrant adherent cells, consistent with previous reports indicating that ethanol impairs neuronal cell adhesion mechanisms and neuronal migration [102, 120]. Tong et al. [174] showed that abnormalities in cerebellar function following chronic prenatal ethanol exposure were associated with inhibition of insulin/IGF, canonical Wnt, and Notch pathways. Thomas et al. [175] showed that neonatal ethanol exposure induces cerebellar Purkinje and granule cell loss if exposure occurs before postnatal day (PD) 7 and that cerebellar damage may underlie ethanol-induced motor deficits. Exposure during PD 4/5 produced significantly more severe motor deficits and significantly more severe reductions in cerebellar and brainstem weights than did exposure later in life.

Another mechanism of disrupted development of the cerebellum involves synaptic defects. A recent study that showed reduced N-acetylaspartate NAA levels in children with PAE using MRS suggests impairment in the early developmental formation of dendritic arborizations and synaptic connections [176]. The study showed that additional finding of lower choline points to disrupted choline metabolism of membrane phospholipids with potentially reduced content of dendrites and synapses. The alcohol-related alterations in glutamate plus glutamine that was identified suggested a disruption of the glutamate–glutamine cycling involved in glutamatergic excitatory neurotransmission.

Fan et al. [177] have confirmed abnormalities in eyeblink conditioning and FASD using the MRI and DTI analyses. Using DTI (which is used to assess integrity of the white matter) they demonstrated a lower response (as measured by fractional anisotropy) bilaterally in the superior cerebellar peduncles and higher diffusivity in the left middle peduncle in the alcohol-exposed children compared to

controls, and the findings correlated with poorer EBC performance. This may reflect poorer myelination in these large bundles of myelinated nerve fibers that connect the cerebellum to the brain stem. The authors conclude that FASD deficits in EBC are likely attributed to poorer myelinization in key regions of the cerebellar peduncles.

Clinical Consequences to Cerebellar Dysfunction in PAE and FASD

Many of the behavioral deficits seen in individuals with FASD, including spatial recognition, motor learning, and fine motor control, are mediated, in part, by the cerebellum [150]. There has been a long-standing recognition and association with cognitive function and cerebellar function [178–181]. Behavioral changes were clinically prominent in patients with lesions involving the posterior lobe of the cerebellum and the vermis, and in some cases they were the most noticeable aspects of the presentation [178]. As noted previously, there is a frequent occurrence of cerebellar defects in autism [182] and also in ADHD children [183]. Berquin et al. [183] showed vermal volume was significantly less in boys with ADHD. This reduction involved mainly the posterior inferior lobe (lobules VIII–X) but not the posterior superior lobe (lobules VI–VII). A cerebello–thalamo–prefrontal circuit dysfunction may subserve the motor control, inhibition, and executive function deficits encountered in ADHD. It is of interest that FASD children frequently present with attention difficulties, and there may be an overrepresentation of autism in PAE and/or FASD children and adults [184].

In a study of children with heavy prenatal alcohol exposure experience, significant deficits in isometric force production were identified that may impede their ability to perform basic motor skills and activities in everyday tasks [185]. In addition, another study results indicated children with FAS experience deficits in response programming and movement time production [186].

Summary

This chapter summarizes select teratogenic agents to illustrate the importance of the recognition of etiology, mechanisms of teratogenesis, pathogenesis, and clinical impact these agents have on the developing human and particularly cerebellar structural and functional consequences. Where appropriate and relevant, the emerging role and effects of genetic and epigenetic mechanisms are discussed. Emphasis has been given to common conditions and hence the greater attention to PAE and FASD. Because of the nature of teratogens, there is opportunity to prevent the occurrence of phenotypic consequences of these exposures through various prevention strategies.

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Primary Pediatric Brain Tumors of the Posterior Fossa: Part I

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Abstract In pediatric neuro-oncology practice, cerebellar tumors are often referred to as infratentorial tumors or tumors of the posterior fossa (a differential diagnosis is provided in Table 1). This anatomic region also contains the pons and medulla, which along with the midbrain comprise the brainstem. In Part I of this comprehensive review, three important pediatric brain tumors usually localized to the cerebellum are discussed (and summarized in Table 2): atypical teratoid/rhabdoid tumors (ATRTs), pilocytic astrocytomas, and ependymomas. In the compan-

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ion chapter (Part II), an integrated clinical and molecular overview of medulloblastoma follows. These tumors have been selected, in part, due to their clinical significance as well as recent advances in their molecular genetics and pathological classification. For these entities and others, the histopathologic, cytogenetic, and molecular factors have been integrated into the updated fourth edition of the World Health Organization (WHO) Classification of Tumors of the Central Nervous System (Louis et al., WHO classification of tumours of the central nervous system, Revised 4th edn. IARC, Lyon, 2016a; Louis et al., Acta Neuropathol 131:803–820, 2016b).

Keywords Childhood brain tumors • Medulloblastoma • Atypical teratoid/rhabdoid tumor • ATRT • Pilocytic astrocytoma • Ependymoma • Posterior fossa • Cerebellum

Introduction

Posterior fossa tumors are located in the infratentorial space that is separated from the supratentorial space by a meningeal fold, the cerebellar tentorium. Important neuroanatomical structures located within the infratentorial space are the cerebellum with the fourth ventricle and caudal part of the brainstem that includes the pons and medulla oblongata. The posterior fossa is the site of a variety of rare primary pediatric brain tumors, including tumors from brainstem glioma, meningioma and schwannoma, hemangioblastoma, hemangiopericytoma, choroid plexus papilloma, and epidermoid cyst (Table 1; [77]). The three most common posterior fossa primary pediatric brain tumors are pilocytic astrocytoma (PA), ependymoma, and medulloblastoma (MB), all of which have associations with the cerebellum. While this chapter (Part I) will discuss pilocytic astrocytoma, ependymoma, and atypical teratoid/rhabdoid tumors (ATRTs), the following chapter (Part II) will focus primarily on MB. All of these tumors share three basic clinical and molecular characteristics (Table 2): (i) Clinical symptoms are caused by posterior fossa compression and occluding hydrocephalus and result in increased intracranial pressure with headaches, progressive nausea, vomiting, lethargy, and drowsiness. Cerebellar tumor location frequently causes ataxia. While these general symptoms do little to differentiate between posterior fossa tumors, children with ependymoma obstructing the foramen of Magendie show distinct torticollis which is rarely observed with other posterior fossa tumors, like MB or PA. (ii) Surgical tumor excision is the initial treatment of choice and, in cases where complete surgical removal is impossible, is combined with targeted radiotherapy, chemotherapy, or both depending on the tumor histology and age of the child. (iii) An emerging common molecular theme is that tumors located at different neuroanatomical locations have distinct cytogenetic/gene expression signatures. This has important implications for the selection of future molecular targets and new therapeutic intervention strategies.

Table 1 Posterior fossa mass: differential diagnosis

Pilocytic astrocytoma
Medulloblastoma
Ependymoma
Atypical teratoid/rhabdoid tumor (ATRT)
Brainstem glioma
Metastatic deposits
Hemangioblastoma
Teratoma
Dermoid cyst
Meningioma
Vestibular schwannoma
Lymphoma
Ganglioglioma
Lhermitte-Duclos disease

Atypical Teratoid/Rhabdoid Tumor (ATRT)

Epidemiology

Atypical teratoid/rhabdoid tumors (ATRTs) are highly aggressive embryonal tumors that predominantly affect very young children. Until recently, this tumor type was thought to be universally fatal [99, 34]. These brain tumors have historically been characterized by their aggressive behavior and poor prognosis, with a median survival ranging from 6 to 11 months [13, 51, 69, 92]. ATRTs are the most common malignant CNS tumor affecting children younger than 6 months of age [22]. Approximately 70% of cases arise in children younger than 1 year of age, and 90% occur before 3 years of age [38], with a median age of 18 months [24].

Overall, ATRTs are estimated to comprise 1–3% of pediatric brain tumors [53, 58], but they account for 20% of CNS tumors in children under the age of 3 years [53]. The CBTRUS data from 2008 to 2012 determined the incidence of ATRT to be 0.34 per 100,000 population in children aged 0–4 years and 0.02 per 100,000 population in children aged 5–9 years [68]. Relative survival estimates for embryonal tumors are low but vary significantly by histology. The current 10-year survival rate for ATRT is 26.5% [68].

SEER data between 1973 and 2010 identified 174 cases of ATRT. There was a significantly higher incidence in males (56.3%), Caucasians (59.1%), and children less than 3 years (80.5%). The most common primary sites were the cerebellum (17.8%), the ventricles (16.1%), and the frontal lobe (12.6%) [53]. In the past,

Table 2 Posterior fossa tumors

	Medulloblastoma	Ependymoma	ATRT	Pilocytic astrocytoma
Age group	Peak incidence 5–9 years	Mean age 6 years	Peak incidence <3 years, median age at diagnosis 18 months	Peak incidence 5–15 years
Gender	M>F (1.6–1)	M=F	M>F (1.5:1)	M=F
Molecular genetics	WNT, sonic hedgehog (SHH), group 3, and group 4	PF-A: epigenetic aberration PF-B: chromosomal aberration	Mutation or inactivation of INI1/hSNF5/BAF47, 90% of tumors have loss of INI1 nuclear staining, indicative of biallelic inactivation of SMARCB1b	>70% of cerebellar PA have *BRAF-KIAA* fusion gene, germline mutations in *NF1* with optic pathway PA
Histopathology	Classic MB, desmoplastic MB, large cell MB, anaplastic MB, and MB with extensive nodularity (MBEN)	WHO grade I–IV, myxopapillary, subependymoma, ependymoma and anaplastic ependymoma	Characterized by rhabdoid cells, small round blue cell tumors	WHO grade I rarely show anaplasia
Management	Maximal safe surgical resection, craniospinal radiation (for those >3 years), and adjuvant chemotherapy	Surgical resection with adjuvant radiotherapy, chemotherapy in young children or patients with residual/recurrent disease	Surgical resection followed by intensive chemotherapy and focal or craniospinal radiation, high-dose chemotherapy with stem cell rescue is also an option	Surgical resection, radiation therapy for progressive disease
Prognosis	10-year survival is 63.3%; 5-year overall survival based on subgroups: WNT (>90%), SHH (~75%), group 3 (40–60%), and group 4 (~75%)	5-year overall survival rate 23–69%	Poor survival, though improving, with median survival of 10–11 months	10-year overall survival rate >90%

ATRT was associated with an extremely poor prognosis, with mean overall survival ranging from 6 to 18 months [69, 96]. SEER data showed a mean overall survival of 3.2 ± 0.4 years, while overall and cancer-specific mortality were 63.2% and 56.3%, respectively. Most ATRT cases were treated with surgery alone (58.0%), followed by a combination of surgery and radiation (34.3%), no treatment (6.5%), and radiation alone (1.2%). However, since 2005, the use of combination therapy has increased significantly (16.1%). The rates of primary surgical resection and radiation therapy remain relatively unchanged. The longest survival has been observed among ATRT patients receiving combination therapy (5.9 ± 0.7 years). Multivariable analysis identified only distant metastases (OR 4.6) as independently associated with increased mortality, whereas combination therapy (OR 0.4) was associated with reduced mortality [53].

ATRTs were first described in the 1987 but were not recognized as a separate tumor entity by the World Health Organization (WHO) until 1993 [48], when they were classified as an embryonal grade IV neoplasm [49]. ATRT is now defined by alterations of either INI1 or, very rarely, BRG1 [30, 44, 106]. These alterations can be evaluated using immunohistochemistry for the corresponding proteins, with loss of nuclear expression correlating with genetic alteration.

Under the revised WHO 2016 Classification, the diagnosis of ATRT requires confirmation of the characteristic molecular defect. If a tumor has histological features of ATRT but does not harbor either of the diagnostic genetic alterations, only a descriptive diagnosis of CNS embryonal tumor with rhabdoid features can be made [57, 59].

Clinical Presentation

ATRTs arise in infratentorial or supratentorial locations in almost equal proportions and rarely arise in the spine [4, 51, 101]. The clinical presentation of ATRT depends on the age of onset and the location of the tumor. Because ATRT grows rapidly, patients typically have a fairly short history of progressive symptoms, measured in days to weeks.

Children younger than 3 years usually present with non-specific symptoms and signs such as vomiting, lethargy, irritability, weight loss, enlarging head circumference, and failure to thrive. Older patients commonly present with increased intracranial pressure or localizing signs. Cranial nerve palsies, headache, and hemiplegia are common [73, 81, 82]. They may also develop ataxia or regression of developmental milestones.

Diagnostic Imaging (Fig. 1)

Among 116 ATRTs in the European Rhabdoid Registry (EU-RHAB), 49% were located within the cerebellum or fourth ventricle, 34% were located in the hemispheres, 4% were located in each of the mesencephalic and pineal regions, 1.7% were found in the spine, and 6% crossed anatomic borders such that origin could not be determined [22].

Imaging features have often been considered non-specific [73, 103]. Parmar et al. [73] demonstrated that lesions are commonly large at presentation, with moderate-to-marked surrounding edema.

In the earlier literature, ATRTs were described as occurring more commonly in the infratentorial region, although this has not been reported in more recent series. Warmuth-Metz et al. [103] described preoperative imaging examinations of 33 patients with ATRT. In their series, supratentorial tumors were more frequent than infratentorial tumors in accordance with some of the largest series evaluating treatment and outcome in ATRT [34, 96]. Supratentorial tumors and those affecting both compartments were significantly larger than those in the infratentorial area. Fifteen percent of their patients showed meningeal dissemination at diagnosis, and this was significantly correlated with a younger age.

Most (52%) of the tumors were surrounded by some edema. Cysts or necrosis was present in 75% of tumors. Cysts in a peripheral position between the solid part of the tumor and the normal brain were seen in 39% patients, with an even distribution between the infra- and supratentorial compartments. This feature seems to be a regular finding in ATRTs [103]. On CT scan, ATRTs are solid or mixed lesions. The solid portion is commonly hyper-dense on non-enhanced CT, a feature attributed to the tumor's high cellularity and high nuclear-to-cytoplasmic ratio [73, 103].

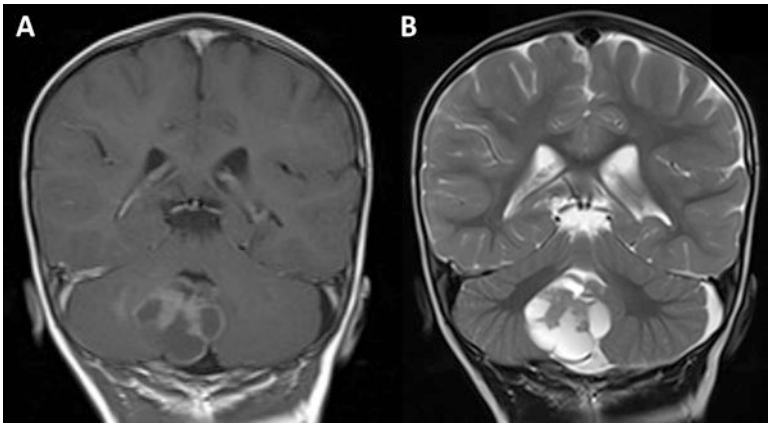

Fig. 1 Seventeen month male with a 3.5 cm atypical teratoid/rhabdoid tumor, localized to the right cerebellar hemisphere with a central solid component and several cystic loculations. (**a**) T1 post-gadolinium. (**b**) T2-weighted image

On MRI, signal intensity values on T1- and T2-weighted MR images vary widely [103]. An example is provided in Fig. 1. Parmar et al. [73] found that greater than 50% of these tumors revealed iso-intensity on T1-weighted images and more than 80% were either hypo-intense or heterogeneous on T2-weighted images. Moderate-to-marked enhancement with gadolinium was seen in all tumors. All intra-axial tumors showed extensive vasogenic edema. Hemorrhage was seen in 46% of patients [73, 103], calcification in 36%, necrosis in 46%, and cysts in 18%. These tumors also had a high propensity for subarachnoid dissemination, with 46% showing the presence of leptomeningeal metastasis at the time of presentation [73].

Parmar et al. [73] recommend that contrast-enhanced MR imaging of the brain and spine should be undertaken at the time of presentation and on follow-up because of the high rate of recurrence and leptomeningeal spread. Similar to most malignancies, ATRT cannot be reliably distinguished from other malignant brain tumors based on clinical history or radiographic evaluation. Surgery is necessary to obtain tissue to confirm the diagnosis of ATRT.

Tumor Pathology (Fig. 2)

Macroscopically, ATRTs are soft, pinkish-red, often well-circumscribed tumors with areas of necrosis and hemorrhages. These tumors arise in the cerebellopontine angle and variably infiltrate cerebellum and brainstem. ATRTs consist of heterogeneous cells with various morphological appearances [81]. Small undifferentiated embryonal cells are the most common tumor cell population, characterized by high nuclear/cytoplasmic ratio. They often contain a vesicular nucleus and a single nucleolus and show less nuclear hyperchromasia than cells of primitive neuroectodermal tumors (PNETs). Small groups or scattered rhabdoid cells typically with eccentrically placed nucleus, large eosinophilic nucleolus, abundant eosinophilic

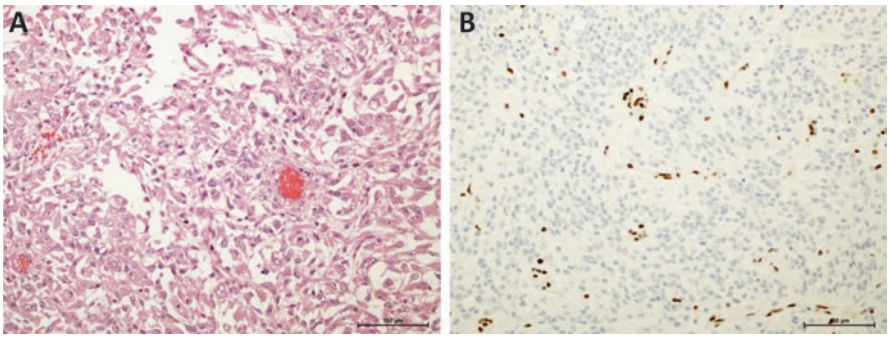

Fig. 2 (**a**) Rhabdoid component of an atypical teratoid/rhabdoid tumor. (**b**) Diagnostic loss of nuclear expression of INI1 in tumor cells, in contrast to INI1 expression in endothelial cells (serve as internal positive controls)

cytoplasm, and eosinophilic globular "ball-like" cytoplasmic inclusion are encountered in most ATRTs (Fig. 2a) but may be absent. In only a minority of ATRTs are rhabdoid cells the predominating component. Cells with glial, neuronal, epithelial, or mesenchymal features are observed in most tumors [63]. Occasionally, multinucleated or pleomorphic giant cells are noted. The mitotic and proliferative index is markedly increased, in particular in pediatric ATRTs. Zonal necrosis and hemorrhage are common. Characteristically, a fine fibrovascular network is present within the tumor, but microvascular proliferation may occur.

By immunohistochemistry, expression of glial fibrillary acidic protein (GFAP), epithelial membrane antigen (EMA), smooth muscle alpha-actin (SMA), and vimentin is found most consistently. Often, small groups or scattered tumor cells are immunopositive for synaptophysin, microtubule-associated protein 2 (MAP2), neurofilament protein (NFP), desmin, cytokeratins, and HMB-45. ATRTs lack nuclear expression of INI1, the SMARCB1 gene product, in contrast to normal tissue ([45]; Fig. 2b) and most other tumors, in particular PNETs, polymorphous gliomas, or rhabdoid meningiomas. ATRTs with retained INI1 expression but with loss of nuclear expression of BRG1, the SMARCA4 gene product, are rare.

Cribriform neuroepithelial tumor (CRINET) also lacks nuclear INI1 expression but shows no rhabdoid tumor component. This rare tumor is morphologically characterized by cribriform strands and trabeculae of epithelial cells [32]. Molecular findings of CRINETs including the methylation pattern are similar to those seen in the molecular ATRT-TYR subgroup, but the morphological appearance of the CRINET tumor cells and the cribriform architecture are different [39]. Diagnosis of CRINETs is important since these tumors may have a favorable prognosis.

Molecular Genetics and Biology

In order to specifically target ATRT with novel therapeutics, it is important to clearly understand the driving molecular mechanisms. In ATRT, recent analysis has elucidated recurring mutations in genes for components of the chromatin remodeling complex, SWItch/sucrose nonfermentable (*SWI/SNF*), in patients with ATRT, with *SMARCB1* being most commonly mutated, followed rarely by *SMARCA4* [22, 31, 55]. Both *SMARCB1* and *SMARCA4* are essential components of the *SWI/*SNF complex, which is important for lineage specification, maintenance of stem cell pluripotency, and gene regulation [38, 39, 104]. The SWI/SNF complex has important functions in neural development [108].

ATRTs are associated with mutation or inactivation of the *INI1/hSNF5/BAF47* tumor suppressor locus on chromosome 22q11.23 in almost all cases [79, 100]. ATRT is characterized by the biallelic loss of *SMARCB1* expression [81]. Up to 35% of patients with CNS rhabdoid tumors have germline *SMARCB1* alterations and a rhabdoid tumor predisposition syndrome characterized by the development of multiple rhabdoid tumors [8, 17, 85]. The majority of germline mutations occur de novo, and transmission across generations is rare [3, 23].

Although findings from small patient cohorts suggest molecular heterogeneity may underlie the clinical spectrum seen in ATRT tumors, cumulative genomic analyses, including whole exome sequencing studies, have shown *SMARCB1* loss as the only recurrent genetic event in ATRT [47, 55]. Reconciling clinical heterogeneity with tumor biology has been challenging, because it is a rare disease and there have been few biological and clinical studies [9], especially ones which studied CNS ATRT independently from non-CNS rhabdoid tumors [99].

Despite the absence of recurring genomic alterations beyond *SMARCB1* (and rarely other SWI/SNF complex members such as SMARCA4), biologically distinctive subsets of ATRT have been identified [38, 39, 99]. Torchia et al. [99] identified two molecular subgroups of ATRT with distinct features. Then, Johann et al. [38, 39] identified three distinctive subsets of ATRT, associated with differences in demographics, tumor location, and type of SMARCB1 alterations through the use of DNA methylation arrays and gene expression arrays [38, 39]. Johann et al. [38] termed these subsets ATRT-TYR, ATRT-SHH, and ATRT-MYC.

The recent transcription and methylation profiling studies by Torchia et al. [99] support the existence of at least two different molecular subgroups, groups 1 and 2 [99]. These tumors may be further stratified into average-, high-, and very high-risk groups by integration of tumor molecular subgrouping and clinical prognostic factors. They defined group 1 ATRTs as those most highly enriched for genes involved in the brain or neural development and axonal guidance and demonstrated upregulation of genes involved in the *NOTCH* developmental signaling pathway. The genes *FABP7* and *ASCL1*, markers of primitive neural lineage, were among the most highly upregulated genes [65, 87]. The *HES5/HES6* and *DLL1/DLL3* genes, which are also involved in the *NOTCH* pathway, were also highly enriched in group 1 ATRTs [14]. Torchia et al. [99] found that as a group-specific marker, *ASCL1* showed robust immunostaining and allowed for distinction between *ASCL1-positive* and *ASCL1-negative* tumors. *ASCL1* expression correlated with superior overall survival (OS), but not with progression-free survival (PFS), for all patients treated with chemotherapy [99].

In group 2 ATRTs, neural lineage marker expression was significantly decreased. Instead, these tumors have enrichment of genes involved in mesenchymal differentiation and the bone morphogenetic protein (BMP) signaling pathway including *BMP4*, *BAMBI*, *SOST*, *SERPINF1*, *FBN2*, and *MSX1* loci [99]. These tumors were significantly associated with infratentorial location, in contrast to group 1 ATRTs which were mostly supratentorial. In a small cohort of patients who did not receive radiation as part of their primary therapy, *ASCL1*-positive group 1 tumors correlated significantly with higher 5-year PFS and 5-year OS relative to the *ASCL1*-negative group 2 tumors. On univariate analysis, it was noted that *ASCL1* expression and not supratentorial tumor location was a significant prognostic factor for both PFS and OS in non-irradiated children [99].

The ATRT-TYR subset represented approximately one-third of cases and was characterized by elevated expression of melanosomal markers such as *TYR* (the gene encoding tyrosinase), *MITF*, or *DCT*. TYR is highly expressed in almost every case in this subgroup, hence the designation *ATRT-TYR*. Cases in this subset were

primarily infratentorial, with most presenting in children aged 0–1 years and 77% showing chromosome 22q loss, which was only seen in 20% and 12% of ATRT-SHH and ATRT-MYCN tumors, respectively.

The ATRT-SHH subset represented approximately 40% of cases and was characterized by elevated expression of genes in the sonic hedgehog (SHH) pathway such as *GLI2* and *MYCN*. Cases in this subset occurred with near equal frequencies in supratentorial and infratentorial regions. While most presented before age 2 years, approximately one-third of cases presented between 2 and 5 years.

The ATRT-MYC subset represented approximately one-fourth of cases and was characterized by elevated expression of MYC. They tended to occur in the supratentorial region. While most ATRT-MYC cases occurred by age 5 years, this subset represented the most common subset diagnosed at age 6 years and older. Focal deletions of *SMARCB1* were the most common mechanism of SMARCB1 loss for this subset.

Despite few differences between the ATRT subgroups at the genetic level, there were remarkable epigenetic differences. Both *ATRT-TYR* and *ATRT-SHH* revealed genome-wide hypermethylation, particularly in promoter regions. *ATRT-MYCN* showed hypomethylation. These differentially methylated regions have a large impact on the expression of genes located within them, including tumor suppressor genes (which are silenced) and oncogenes (which are activated) in regions where the partially methylated domain is absent. *SMARCB1* expression should be evaluated in all young patients with embryonal tumors to confirm the diagnosis of ATRT rather than medulloblastoma or other CNS PNETs.

Therapy and Prognosis

Survival rates for patients with ATRT are generally poor but have improved over recent years due to the development of clinical trials specifically designed for ATRT with stringent inclusion and exclusion criteria and a renewed focus on the vulnerability of affected young patients [28]. To date, no standard of therapy for ATRT has been defined. A significant proportion of ATRTs arise in children younger than 3 years. Treatment with conventional postoperative chemotherapy alone results in less than 20% survival [27, 29, 96]. Small cohorts of patients treated with ATRT-specific regimens have achieved survival rates greater than 50% [13, 96]. Improved survival has also been demonstrated for patients with gross total resection [13, 51].

Most recent treatment strategies recommend maximal safe surgical resection followed by intensive chemotherapy with or without intrathecal chemotherapy and focal or craniospinal radiation. However, treatment depends on the location of the tumor, initial staging, and age of the patient at presentation. The management of ATRT with conventional chemotherapy has been consistently associated with very poor outcomes, and most series have supported the benefit of aggressive multimodal therapy [51, 78]. While a multimodal approach that combines maximal safe resection, craniospinal irradiation, and intensive chemotherapy is considered optimal for long-term cure, the young age of many patients and/or involvement of critical structures within the CNS limits this approach [38, 39, 91].

In recent years, treatment approaches in Canada have been more homogeneous and based on the use of high-dose chemotherapy [51]. Treatment factors that predict survival have included the use of multimodality regimens containing radiotherapy, intrathecal chemotherapy, and/or high-dose therapy with stem cell rescue [4, 13, 26, 51, 96]. The series of patients investigated by Lafay-Cousin et al. [51] highlights the encouraging results associated with the use of high-dose chemotherapy and describes a proportion of long-term survivors (50%) who did not receive radiation. Novel therapy that improves outcomes while it decreases toxicity is greatly needed. As ATRT is typically a tumor of infancy, radiation-free approaches are often used in patients to minimize long-term neurodevelopmental sequelae [99]. Current curative therapy for ATRT is perhaps excessively toxic, including the acute toxicity of high-dose chemotherapy [26] and long-term toxicity of radiotherapy in young children. A major focus of current research is on the development of more focal, and potentially less harmful, methods of radiotherapy, such as proton beam radiation.

Data from a small cohort by Torchia et al. [99] suggests that children with localized supratentorial ATRT, with high *ASCL1* expression and complete surgical resection, represented a favorable-risk category with a projected 5-year PFS and OS of 60%, with disease recurrence in only about 33% of patients [99]. This will have to be validated in future trials. Ongoing, prospective studies will more precisely define the outcome of children with ATRT in the current era.

Future Considerations

The availability of ATRT cell lines and accurate preclinical mouse models have enhanced the discovery of novel therapeutic targets for ATRT. Current targets under consideration are aurora A kinase, cyclin D1, EZH2, and insulin-like growth factor-1. The availability of aurora A kinase inhibitors has facilitated the development of a phase II trial for patients with recurrent ATRT and malignant rhabdoid tumor (NCT02114229). If this trial demonstrates the efficacy of this agent, it will most likely be incorporated into therapy for patients with newly diagnosed ATRT [24]. Results from Torchia et al. [99] suggest that inhibitors of *NOTCH*, *BMP*, and MAPK signaling and angiogenesis would be important novel, subgroup-specific therapeutic agents for ATRT [99].

Pilocytic Astrocytoma

Epidemiology

Pilocytic astrocytomas (PAs) are a distinct histologic and biologic subset of gliomas and account for 5% of all gliomas. PAs are typically well-circumscribed WHO grade I tumors that have a slow growth rate. PA is the most common primary brain tumor in 0- to 19-year-olds. Pilocytic astrocytoma accounts for 15% of children and adolescents (0–14 years) and 18% of childhood (0–14 years) primary brain tumors [10].

Clinical Presentation

Pilocytic astrocytomas arise throughout the CNS, although most frequently occur in the cerebellum (42%), followed by the supratentorial compartment (36%), the optic pathway and hypothalamus (9%), the brainstem (9%), and the spinal cord (2%) [10]. A rare variant termed "pilomyxoid astrocytoma" occurs predominantly in children under 1 year of age, in the hypothalamic/chiasmatic region. Pilomyxoid astrocytoma was categorized as WHO grade II in the 2007 WHO Classification due to reports of an increased likelihood of recurrence, but tumor grading for this entity has been omitted in the 2016 update [57, 59].

The presentation of PAs is generally insidious in onset due to the slow growth of the tumor. Identification of early symptoms is dependent on tumor localization and the ability of the patient to communicate neurological change. Cerebellar tumors commonly present with ataxia, cranial nerve defects, and signs of increased intracranial pressure (headache, nausea, and vomiting).

Diagnostic Imaging (Fig. 3)

Neuroimaging in PA is used to determine the size and the site of origin of the lesion, establishing a primary diagnosis. PA is easily imaged on both CT and MR imaging. On CT images, PAs classically present as a mass with both a solid and cystic component. The solid component usually enhances with contrast, and the cyst wall has variable enhancement. The appearance of a cyst with a mural nodule is almost pathognomonic for PA. On MR imaging, the cystic and solid components are better appreciated. PAs are typically hypo- or iso-intense on T1-weighted sequences and hyperintense on T2-weighted or FLAIR sequences ([2]; Fig. 3).

Tumor Pathology (Fig. 4)

Pilocytic astrocytomas (PAs), WHO grade I, are macroscopically soft, gray, often mucoid, and well-demarcated tumors. Many cerebellar PAs form cysts within or adjacent to the tumor, with a contrast-enhancing solid mural nodule, similar to hemangioblastomas and gangliogliomas. These cysts contain clear, yellow, or brown protein-rich fluid and are often demarcated by a compressed tumor area with variable fibrous changes.

Histopathologically, PAs characteristically have a biphasic architecture, composed of a loosely textured microcystic and a compact fibrillary component (Fig. 4a). The microcystic component contains astrocytes with short multipolar process, whereas the astrocytes of the fibrillary component have uni- or bipolar hairlike ("piloid") processes. Rosenthal fibers, amorphous sausage-like eosinophilic struc-

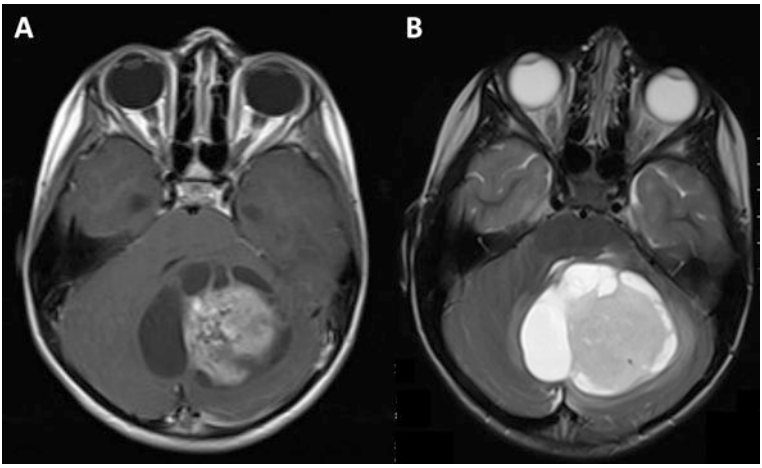

Fig. 3 Four-year-old with a large 5.4 × 5.8 × 5.2 cm mass, a pilocytic astrocytoma located in the left cerebellum with extension across the vermis into the medial aspect of the right cerebellar hemisphere. (**a**) T1 post-gadolinium. (**b**) T2-weighed image

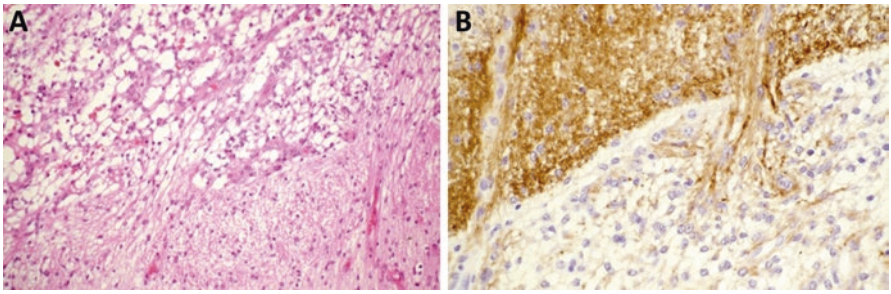

Fig. 4 (**a**) Pilocytic astrocytomas are characterized by a biphasic tumor architecture, with a solid fibrillary and a loose microcystic component (H&E). (**b**) Strong expression of the astrocytic marker glial fibrillary acidic protein (*GFAP*) is present in bipolar tumor cells of solid fibrillary areas in contrast to multicystic tumor cells of microcystic areas

tures, are more frequent in the fibrillary component but may be absent. Eosinophilic granular bodies (EGBs), proteinaceous material positive in periodic acid Schiff (PAS) stain, are present in the multicystic component of some PAs. Both structures can be found in other neoplasms and in nonneoplastic lesions. Many PAs are rich in vasculature, most often hyalinized vessels are present, but serpentlike microvascular proliferations with glomeruloid vessels are also frequent. Often, these glomeruloid proliferations are lining the tumor cyst wall. Some classical PAs show zonal, ischemic-like necrosis. Neither the presence of necrosis, of microvascular proliferations, nor of degenerative features such as nuclear hyperchromatism, pleomorphism, and pseudoinclusions indicate a worse prognosis. Rare mitotic figures may be present in classical PAs. Diffuse brisk mitotic activity, usually defined as >4 mitotic

figures per 10 high-power fields, indicates anaplastic change and has prognostic implications [80]. Necrosis is often present but not associated with anaplasia. The prognosis of these *anaplastic pilocytic astrocytomas* is better than in glioblastomas.

Classical PAs are well-circumscribed tumors which typically show only focal infiltration of surrounding brain tissue. In contrast, some PAs mimic diffusely infiltrating astrocytomas by morphology, tumor architecture, and infiltration behavior but have a much better prognosis than diffusely infiltrating astrocytomas. This *diffuse "variant" of pilocytic astrocytomas* (dPAs) has a similar prognosis compared to classical PAs [33], and approximately 50% harbor the most common BK fusion variant [35]. Thus, molecular findings and biological behavior suggest that classical PAs and dPAs represent a single tumor entity. Diffuse astrocytomas account for approximately 15% of all cerebellar astrocytic tumors, but most are high-grade astrocytomas. Particularly cerebellar PAs often show infiltration of leptomeninges with focal desmoplasia, a finding that does not predict subarachnoid dissemination or CSF spread and does not affect prognosis.

Pilomyxoid astrocytoma (PMA) is typically found in the hypothalamic region but rarely occurs in cerebellar location. PMA is characterized by monomorphous bipolar tumor cells, often in angiocentric arrangement, and myxoid tumor matrix [97]. PMAs are associated with a more aggressive clinical course; thus, these tumors were assigned to WHO grade II in the 2007 CNS tumor classification. However, the tumor grade for PMA has been reconsidered in the subsequent upgrade [57].

PAs characteristically show strong immunoreactivity for glial fibrillary acidic protein (GFAP), S100, and OLIG2. The bipolar tumor cells of compact areas are strongly immunopositive for GFAP, whereas multipolar tumor cells show weaker expression (Fig. 4b). Rosenthal fibers are often GFAP immunopositive in their fibril-rich periphery. Weak expression of synaptophysin may be present in occasional PAs and PMAs.

Molecular Genetics and Biology

Molecular classification of PAs has been slowly evolving since 2008. High-throughput genetic sequencing and gene expression profiling have made information regarding the biologic processes necessary for tumor growth and a molecularly based approach to therapy possible. Alterations in the RAS/RAF/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway are found in the majority of PAs [40, 93].

The most common genetic alteration found in PAs is the tandem duplication at 7q34, which produces a fusion between two genes, *BRAF* and *KIAA1549*. This "B-K" gene fusion occurs in up to 70% of PAs and is most frequent in cerebellar tumors (72–98%) and less frequent in the other sites such as the optic pathway (43–69%) [6, 7, 36, 40]. The N-terminal end of KIAA1549 replaces the N-terminal end of BRAF, producing a constitutively activated BRAF kinase domain and activa-

tion of the Ras/ERK pathway [7, 21, 42, 89]. The fusion protein can be derived from at least nine different fusion site combinations, with the most common fusion between *KIAA1549* exon 16 and *BRAF* exon 9 [41, 110]. Other gene fusions leading to constitutively active BRAF protein fusion products have also been described in PAs, including FAM131B, RNF130, CLCN6, MKRN1, GNA11, QK1, FZR1, and MACF1 [15, 21, 41, 75, 110].

Activating gene mutations have also been described in a subset of PAs (2–9%). The $BRAF^{V600E}$ mutation results in constitutively active BRAF protein. This mutation, unlike the *KIAA1549-BRAF* fusion protein, is *not* specific to PAs [7, 43, 84, 89]. The *BRAFinsT* mutation has also been described in PA in up to 3% of tumor samples specifically in the young adult population [18].

Multiple additional genetic alterations have been described in PAs. *KRAS* somatic mutations occur at low frequency (3–5%) [16, 24]. Aberrations affecting *FGFR1*, including point mutations (P.N546K, P.K656E), FGFR1-TACC1 fusions, and internal tandem duplications, have been identified [41, 90, 110]. NTRK family receptor kinase mutations have also been reported at a low rate, due to gene fusions leading to kinase activation [41, 110]. FGFR1 alterations are more frequent in midline structures, whereas BRAF V600 and NTRK family fusions are more frequent in supratentorial tumors [52, 88, 95]. There is unknown significance of reported 41% of PAs having MYB protein upregulation. Genomic alterations of MYB have only been found in diffuse gliomas [93].

PAs, particularly optic pathway tumors, occur in up to 20% of neurofibromatosis type 1 (NF1) patients. NF1 is an autosomal dominant syndrome due to mutations in the *Nf1* tumor suppressor gene leading to an increase in the active form of Ras and constitutive activation of the Ras/ERK signaling pathway [5, 16, 54, 55, 86]. In general, patients with NF1-associated PAs have a more indolent course and are less likely to require treatment.

Epigenetic analysis revealed a hypomethylation signature specific to PA that included many differentially methylated developmental genes and suggests aberrant expression of developmental regulatory processes as a genetic cause of PA [37, 52]. Both transcriptome and methylome analyses revealed a distinctive pattern for infratentorial versus supratentorial PA [52, 88, 95].

Therapy and Prognosis

Overall, PA has an excellent prognosis with 10-year survival over 90% [10]. The treatment is primarily surgical, and prognosis depends on the completeness of the resection. Patients who undergo subtotal resection are often treated with chemotherapy and/or radiation therapy at tumor progression to improve long-term survival. Chemotherapy following low-grade glioma protocols is the preferred option for younger patients due to the long-term sequelae of radiation in the developing neuroaxis [76]. Infrequently major postoperative sequelae occur, such as postoperative posterior fossa mutism syndrome (<5% of patients) or marked new brainstem

or cerebellar deficits [66]. More commonly, mild fine motor or balance issue occurs but often does not interfere with activities of daily living. Long-term survivors usually have close-to-normal academic achievement, and measures of quality of life are usually normal [1, 111].

Future Considerations

Although targeted therapies are unlikely to become the standard of care for newly diagnosed PA, identification of the BRAF V600E mutation suggests a poorer prognosis, and these tumors may respond to BRAF inhibitor therapies, such as with vemurafenib and dabrafenib [11]. Furthermore, since resistance to BRAF inhibitors is often encountered, combination with a MEK inhibitor may be beneficial [20]. Future clinical trials will incorporate molecular genetic tumor profiling, and targeted therapies will be carefully integrated [70].

Ependymoma

Epidemiology

Ependymomas are primary tumors in the CNS and account for 10% of childhood brain tumors and about 30% of tumors in children less than 3 years of age [58, 61]. The majority of ependymomas are seen in children less than 7 years old, with 25–51% of cases in children under 3 years of age. A second peak is observed in adults in the third to fifth decades, although the histologic subtypes and neuroanatomic compartments vary considerably between children and adults. Ependymomas originate from the radial glial stem cells and therefore can occur at any site along the ventricular system and in the spinal cord [94]. The anatomical distribution varies according to age, supratentorial compartment and spinal cord being more common sites in older children and adults, with infratentorial locations more frequent in infants and children [58]. Overall, supratentorial tumors account for one-third, whereas posterior fossa tumors, including the cerebellum, account for two-thirds of ependymomas.

Clinical Presentation

The presentation of ependymoma depends on the location of the tumor, and often, due to slow growing nature of the tumor, onset of symptoms and signs can be insidious. Posterior fossa lesions present with symptoms of raised intracranial pressure,

such as headache, nausea and vomiting, ataxia, vertigo, and papilledema. Cranial nerve palsies are also common, involving cranial nerves VI–X. When tumors arise in the supratentorial compartment, seizures or focal neurologic deficits may be present. Tumors involving the spinal cord present with deficits due to compression of nerve roots or ascending/descending nerve tracts and are related to the anatomical level of the tumor.

Diagnostic Imaging (Fig. 5)

Imaging in ependymomas, similar to other CNS tumors, is used to establish a primary diagnosis and determine the size and site of origin of the lesion. Ependymoma can be imaged using both CT and MRI. On CT, the tumors are usually isodense to the brain parenchyma and may have calcifications in up to 50% of cases [12]. On T1-weighted MR imaging, ependymomas are usually hypo-intense or iso-tense to normal gray matter and heterogeneously enhance after contract administration. On T2-weighted images, they are typically isodense or slightly hyperintense to normal gray matter. Foci of signal heterogeneity representing methehemoglobin, hemosiderin, necrosis, calcification, encased native vessels, or tumor vascularity are commonly seen [12] (Fig. 5). It is important to image the entire craniospinal axis, as neuroaxis dissemination can occur in 3–11% of cases [74].

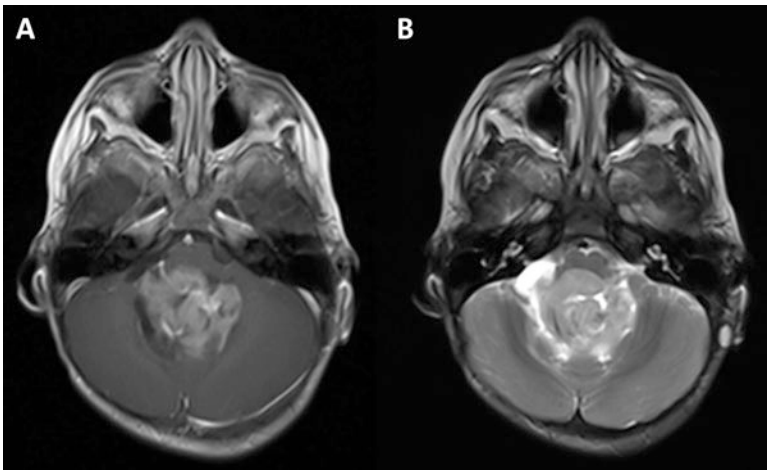

Fig. 5 Fifteen month male with ependymoma with cystic and solid components localized to the fourth ventricle measuring 3.9 × 3.4 cm. (**a**) T1 post-gadolinium image. (**b**) T2-weighted image

Tumor Pathology (Fig. 6)

Ependymomas are well-circumscribed, soft, occasionally cystic, tan-colored tumors that most often arise from the fourth ventricle in the posterior fossa. Commonly, they extend through the foramina of Luschka and Magendie into the cerebellopontine angle and basal cisterns where they often enclose cranial nerves and vessels.

Histopathologically, ependymal tumors are sharply demarcated and present with a wide spectrum of cell morphology but share key features: pseudorosettes are perivascular arrangements of tumor cells which fibrillary cell processes create perivascular anuclear zones (Fig. 6a). True ependymal rosettes are composed of mostly cuboidal tumor cells with a central lumen.

Classic ependymoma (WHO grade II) is characterized by small uniform tumor cells with round-to-oval nuclei in variable cell density. In some ependymomas, nodules of high tumor cell density are present, often associated with an increased mitotic activity. Pseudorosettes are a typical feature of ependymomas, whereas true ependymal rosettes are seen in ~25%. Hemorrhages and dystrophic calcifications are often observed. Other morphological variants include *papillary*, *clear cell* [83], and *tanycytic ependymomas* (WHO grade II) which occur less often in the posterior fossa.

Anaplastic ependymoma (WHO grade III) is defined by a high cell density, high mitotic activity, microvascular proliferation, and necrosis, but the association between histological grade and clinical outcome is controversial. Age of the patient and anatomical site of the tumor appear to be more reliable prognostic factors in ependymomas.

Immunohistochemically, the vast majority of ependymomas express S100, vimentin, glial fibrillary acidic protein (GFAP), and epithelial membrane antigen (EMA). Expression of GFAP is typically present on the luminal surface of true ependymal rosettes and in the perivascular anuclear zones of pseudorosettes. Many ependymomas show dot- or ringlike cytoplasmic immunopositivity for EMA (Fig. 6b). In contrast to supratentorial ependymomas, expression of L1CAM, which indicates rearrangement of *C11orf95*, is not detectable in posterior fossa ependymomas.

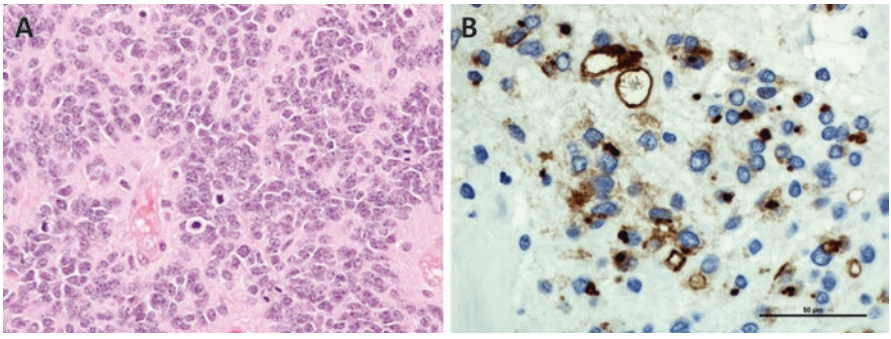

Fig. 6 (**a**) Anaplastic ependymoma, WHO grade III, with markedly increased mitotic activity. Five mitotic figures are seen in this high-power field. (**b**) Cytoplasmic dot- and ringlike immunoreactivity for epithelial membrane antigen (EMA) in an anaplastic ependymoma

Molecular Genetics and Biology

Risk stratification based on histological categorization is difficult in ependymomas, and variability has been seen in outcomes despite similarities in microscopic characteristics. Therefore, molecular analysis has been undertaken to elucidate the pathogenesis of these tumors. In genomic studies, supratentorial ependymomas have been found to have genomic clustering in the region of chromosome 11q12.1–q13.3. This region undergoes gross interchromosomal and intrachromosomal rearrangements, leading to the fusion of the poorly characterized gene *C11orf95* and *RELA*, a downstream target of NF-κB, an important regulator of cell maintenance. This rearrangement has been found in up to 70% of supratentorial ependymomas [72]. A second recurrent gene fusion product, *Cllorf95* and *YAP1*, has also been described predominantly in the younger age group and appears to have a favorable survival outcome, although further studies need to occur to elucidate its role in tumorigenesis [71].

Posterior fossa ependymomas have also been studied in genomic analyses, leading to the transcriptional profiles of posterior fossa (PF) group A (PFA) and group B (PFB) ([105]; Table 3). PFA patients are usually younger, with tumors located laterally and extending to the cerebellopontine angle. Overall PFA tumors are more aggressive in nature and are associated with poor outcomes. These tumors demonstrate relatively stable cytogenetics, although up to 25% of PFA ependymoma has a gain of chromosome 1q, correlating with a poor prognosis [60, 71]. Upregulation of multiple cancer-related signaling pathways has been observed, although they are not specific to ependymomas, including PDGFR, EGFR, VEGF, MAPK, and TGFβ [102, 105]. Epigenetic modification, specifically hypermethylation, has also been demonstrated in PFA tumors. The genes that are CpG methylated in PFA ependymomas are similar to the genes that are silenced by the polycomb repressive complex 2 (PRC2) in embryonic stem cells. PRC2 controls all forms of methylation of lysine 27 on histone H3 and is responsible for silencing genes involved in cell differentiation and tumorigenesis [19, 67].

PFB ependymomas often arise in older patients, occur more frequently in the midline, and are less likely to metastasize. PFB tumors demonstrate greater copy number variation with gain of chromosome 9, 15q, and 18 or loss of chromosome 6q and 22q. These cytogenetic abnormalities have been associated with improved

Table 3 Posterior fossa ependymoma summary

	PF-A	PF-B
Age group	Children	Adults and older adolescents
Gender	M>F	M<F
Prognosis	Poor	Good
Molecular genetics	Epigenetic modification, LAMA-2 expression	Chromosomal modification, NELL2 expression
Histopathology	Anaplastic ependymoma, WHO grade II/III	Anaplastic ependymoma, WHO grade II/III

prognosis [50, 105]. PFB ependymoma does not demonstrate the epigenetic modifications and hypermethylation profiles when compared to PFA tumors.

An unsupervised gene clustering and multivariate analysis revealed a 10-gene signature that qualified as an independent predictor of recurrence-free survival in infratentorial ependymoma [102]. As a result of these key discoveries, novel therapeutic strategies can now be tested that target PRC2 and alter DNA methylation status in ependymoma [25].

Therapy and Prognosis

Overall the prognosis for ependymoma is relatively unsatisfactory with overall survival reported as 50–71%. Local control with surgical resection is clinically important as ependymoma is often locally invasive with low metastatic potential. Leptomeningeal dissemination is seen at diagnosis in only 7–12% of cases, and recurrent disease most frequently occurs at the primary tumor site [64]. Survival of patients with GTR ranges from 66% to 80%, compared to subtotal resection survival of 0–47%. Unfortunately, GTR can only be achieved in approximately 50% of cases due to tumor location and risk of unacceptable neurological injury, often requiring patients to be managed with a tracheostomy and/or gastric feeding tubes [62]. Postoperative involved field radiation therapy is standard of care for patients older than 1 year with non-disseminated ependymoma to lower the risk of local recurrence. Many children in the USA and other countries are being referred to treatment centers that offer proton radiotherapy instead of the more widely available photon-based delivery systems. The role of chemotherapy is less well established and is being investigated in clinical trials. The goal of chemotherapy is to defer radiation therapy in younger patients and as an adjunct for patients with residual disease to improve overall survival [56]. However, chemotherapy has not made a significant impact in this disease [46]. Relapsed ependymoma has an extremely poor prognosis with 5-year overall survival rate reported at 28%, with the median time to recurrence or progression distributed at 18–45 months [98, 109].

Future Considerations

The impact of chemotherapy as a therapeutic strategy to delay radiotherapy or permit “second look” surgery remains unclear. Although several phase II studies offering EGFR inhibitors and/or other receptor tyrosine kinase inhibitors to patients with recurrent, progressive ependymomas have been completed, results have been less than promising. Given the more common presentation of the genomically “bland” PFA tumors in childhood, epigenetically based therapies may hold more promise [46, 107].

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Primary Pediatric Brain Tumors of the Posterior Fossa Part II: A Comprehensive Overview of Medulloblastoma

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Abstract Medulloblastoma (MB) is the most common malignant primary brain tumor in children and is currently classified into five distinct molecular subtypes (WNT, SHH-*TP53* wild type, SHH-*TP53* mutant, Group 3, and Group 4), based on genomic alterations, gene expression profiles, responses to treatment, and developmental cell of origin. The standard treatment for MB consists of surgical

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resection followed by radiation therapy and chemotherapy. However, current treatments do not take into account the extensive heterogeneity between and within MB subtypes. Cancer stem cells also play an important role in treatment failure and recurrence in MB, adding an additional layer of complexity in the form of cellular heterogeneity. This chapter will focus on the clinical presentation of MB, current treatment options, and histological classifications with a more detailed description of the current molecular subtypes, followed by exploration of cellular heterogeneity in the molecular era. Further dissection of tumor heterogeneity and identification of subtype-specific biomarkers will be crucial in the development of novel diagnostic markers and targeted therapies for these highly aggressive pediatric brain tumors.

Keywords Posterior fossa tumors • Medulloblastoma • Pediatric • Tumor heterogeneity • Cancer stem cell

Medulloblastoma (*MB*) is the most common form of primary malignant pediatric brain cancer in North America [1]. Primary MB tumors typically develop in the cerebellum and fourth ventricle; however, extensive dissemination through the cerebrospinal fluid (CSF) often leads to metastasis and tumor recurrence [1]. MB is considered to be a highly heterogeneous disease consisting of a mixture of malignant cells that display distinct genetic, molecular, and cellular characteristics including differences in morphology, gene expression profiles, genetic abnormalities, cellular differentiation, proliferation, response to therapy, and metastatic potential [1]. Extensive heterogeneity exists not only between tumors (intertumoral heterogeneity) but also within tumors (intratumoral heterogeneity). It is now known that tumor heterogeneity plays an important role in treatment failure and recurrence in brain tumors.

Clinical Presentation

MBs typically arise in the cerebellum, with tumor cells filling the fourth ventricle, resulting in obstructive hydrocephalus [2]. Due to the rapid growth of MBs, patients generally have a short duration of symptomatology prior to presentation, ranging from 1 to 3 months [3, 4]. Signs and symptoms can be divided into four categories:

(a) *Increased intracranial pressure*

Some degree of hydrocephalus and increased intracranial pressure is present in nearly 80% of patients at the time of diagnosis. Initial nonspecific headaches are frequently followed by more severe headaches, especially morning headaches, nausea, and vomiting. Papilledema is often present at diagnosis. In infants and very young children, the clinical presentation may not be classical. Instead, they may present with papilledema, vomiting, irritability, delayed closure of the fontanelles or bulging of the anterior fontanelle with open sutures,

and increasing head circumference. Due to dilatation of the third ventricle, very young children are also more likely to have paralysis of upward gaze, a phenomenon known as "sun-setting" eyes [2]. In older patients, especially adolescents and young adults, MBs tend to be located more laterally, involving the lateral cerebellar hemispheres and/or the cerebellopontine angle. With a more lateral presentation, hydrocephalus is slightly less common [2]. Headaches may be nonspecific for 2–5 months before the tumor becomes large enough to cause CSF obstruction and localizing cranial nerve deficits, including unilateral sixth, seventh, and eighth cranial nerve dysfunction; less commonly, hoarseness and swallowing difficulties due to lower cranial nerve dysfunction may be present at the time of diagnosis.

(b) *Localizing signs*

Midline cerebellar deficits are also common, including truncal and gait ataxia. Head tilt may be present due to cerebellar tonsillar herniation, with some associated neck rigidity [2]. Infrequently, in midline tumors, other cranial nerve deficits, presenting as facial weakness, hoarseness, or swallowing difficulties, may be present.

(c) *Non-localizing signs*

Subjective diplopia (double vision) occurs in less than 50% of patients and is most commonly due to a non-localizing abducens nerve palsy [2].

(d) *Signs of metastatic disease*

Although as many as 30% of patients with MB will have disseminated disease at the time of diagnosis, symptoms attributed to leptomeningeal dissemination are relatively infrequent [2]. Occasionally, children who have disseminated disease at diagnosis will complain of neck and back pain relatively early in the course of their illness.

Current Treatment and Traditional Therapies

The standard therapy for MB consists of surgical resection, followed by radiation therapy (RT) and chemotherapy depending on the age of the patient [5]. Risk stratification and treatment regimens over the past 20 years have been determined by the presence of metastasis, extent of resection at diagnoses, and age of the patient [6]. Based on these criteria, patients are stratified into three treatment groups. The first treatment group consists of children greater than 3 years of age with standard risk disease. These patients have total or near-total resection of their tumors and no evidence of dissemination in the CSF [6, 7]. The second treatment group consists of children greater than 3 years of age with high-risk disease, defined as the presence of greater than 1.5 cm^2 of residual tumor after surgery or dissemination/metastasis with large cell/anaplastic histology. Patients with high-risk disease are at an increased risk for tumor recurrence or death compared to the average-risk disease group [6]. Lastly, children younger than 3 years of age constitute a separate treatment

group. Although RT can improve disease control and is typically used for MB patients over 3 years of age, it is not recommended for children under 3 years old. These patients are treated with high-dose chemotherapy to delay or remove the need for RT and allow the nervous system an opportunity to further develop [8].

Histological Classification

Traditionally, MB has been classified based on histological properties. MB appears as a small round blue cell tumor, a characteristic seen upon hematoxylin and eosin (H&E) staining attributed to the presence of large nuclei and scant cytoplasm in less differentiated cells [9]. MB can be divided into four main histological variants: *classic, large cell/anaplastic* (*LCA*)*, desmoplastic/nodular, and MB with extensive nodularity* (Fig. 1) [10].

Classic histology accounts for about 70% of MBs and is characterized by the presence of small, round, undifferentiated cells with a high nuclear-to-cytoplasmic ratio and hyperchromatic nuclei (Fig. 1a). Approximately 40% of classic MBs exhibit Homer Wright rosettes, circular nuclear arrays with fine tangled cytoplasmic processes [10]. Most classic MBs express, at least focally, neuronal antigens such as synaptophysin, class III beta-tubulin, or microtubule-associated protein 2 (MAP2). GFAP expression in the undifferentiated tumor cells is demonstrated in approximately 10% of classic MB. In some classic MB, no expression of neuronal or glial

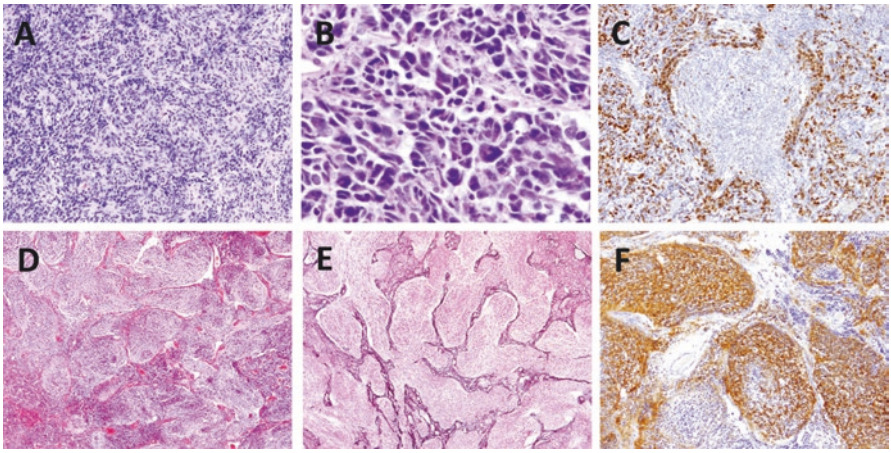

Fig. 1 (**a**) Classic medulloblastoma, WHO grade IV, hematoxylin and eosin (H&E). (**b**) Large cell/anaplastic medulloblastoma, WHO grade IV (H&E). (**c**) Desmoplastic/nodular medulloblastoma, WHO grade IV, MIB1 proliferation marker (Ki67). (**d**) Medulloblastoma with extensive nodularity, WHO grade IV (H&E). (**e**) Medulloblastoma with extensive nodularity, WHO grade IV, reticulin stain. (**f**) Medulloblastoma with extensive nodularity, WHO grade IV, synaptophysin immunostaining

antigens is observed. A strong relationship is observed between *CTNNB1* (encoding β-catenin) mutations and nuclear accumulation of β-catenin, which is indicative of WNT pathway activation, in classic MB [11]. Nuclear accumulation of β-catenin is associated with excellent clinical outcomes in these tumors [11, 12].

Large cell/anaplastic (*LCA*) MB is the most malignant histological variant and is characterized by nuclear pleomorphism with large nuclei, prominent nucleoli, and abundant cytoplasm (Fig. 1b) [10, 13]. Most large cell MBs are heterogeneous and contain intermingled areas of anaplastic and/or classic tumor cells. Expression of synaptophysin is detected in nearly all tumors, whereas neurofilament protein or chromogranin may not be found by immunohistochemistry. GFAP expression is rare. Cell-cell wrapping is a typical feature of anaplastic MBs, with the engulfed cell often undergoing cannibalistic cell death.

Anaplastic MBs consist of tumor cells with enlarged pleomorphic nuclei and distinctive nuclear molding [10, 13, 14]. The nuclear-to-cytoplasmic ratio is high. Mitotic, proliferative, and apoptotic indices are high, comparable to those in large cell MBs. Cell-cell wrapping is a typical feature of anaplastic MBs, with the engulfed cell often undergoing cannibalistic cell death (Fig. 1b). The anaplastic tumor component should be the most prominent to diagnose this rare histopathological MB subtype. The combination of large cell and anaplastic components is often seen and accounts for approximately 10% of all MBs.

Desmoplastic/nodular MBs consist of nodules of differentiated neurocytic cells surrounded by internodular, reticulin-rich zones (Fig. 1c). The internodular zones contain densely packed, highly proliferative cells [10]. Immunohistochemically, MIB-1 labeling index is higher in internodular zones, reflecting the higher mitotic activity of the typically undifferentiated cells present in these areas [10]. Expression of the neuronal markers synaptophysin or NeuN is variable, whereas GFAP expression can be detected only in some desmoplastic/nodular MBs, preferentially in internodular zones. Rare desmoplastic/nodular MBs show nuclear accumulation of p53, and this may indicate an underlying somatic or germline mutation in *TP53*.

MB with extensive nodularity is characterized by an expanded nodular or lobular architecture with elongated reticulin-free, neuropil-rich zones containing uniform neurocytic tumor cells [15] (Fig. 1d). Frequently, no mitotic activity of these cells is detected. The neurocytic cells often exhibit a streaming pattern. The internodular zones may be reduced compared to desmoplastic/nodular MBs but show the same characteristic features of undifferentiated, proliferating tumor cells within a dense reticulin network (Fig. 1e). By immunohistochemistry, neurocytic cells show a strong expression of synaptophysin (Fig. 1f) or NeuN.

Emergence of the Medulloblastoma Molecular Subtypes

Despite the existence of histopathological subtyping, treatment regimens for all MB patients are currently based on metastatic stage and age at the time of diagnosis. It was therefore necessary to develop a new risk stratification system that could

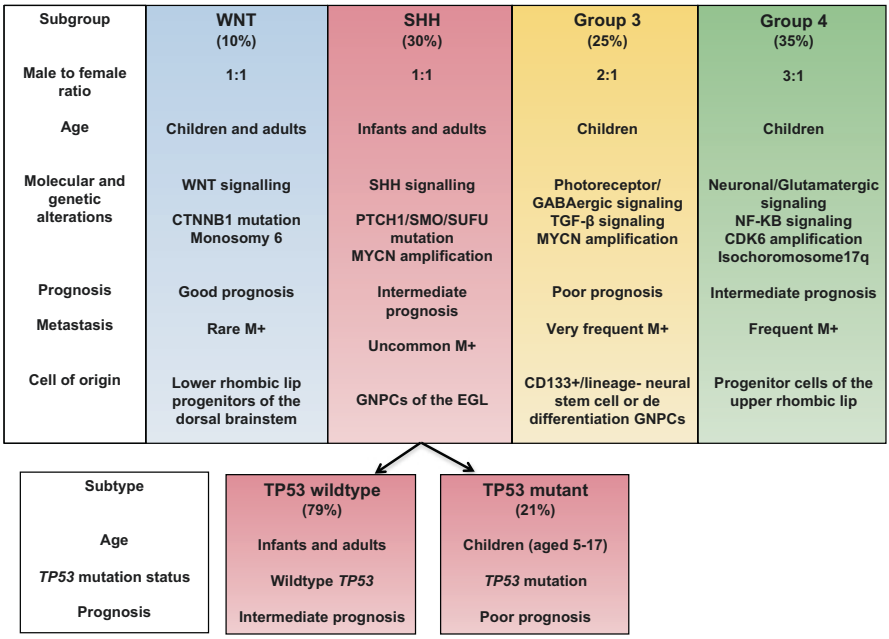

Fig. 2 Medulloblastoma molecular subtypes. There are five molecular subtypes of MB (WNT, SHH-*TP53* wild type, SHH-*TP53* mutant, Group 3, and Group 4) that exhibit difference in demographics, molecular/genetic alterations, prognosis, and cell or origin. WNT and SHH MBs are characterized by dysregulations in WNT and SHH signaling pathways, respectively, whereas less is known about the genetic and molecular drivers of Group 3 and 4 MBs. Due to extensive heterogeneity even within the subtypes, SHH-activated tumors have been recently further divided into SHH-*TP53* wild type and SHH-*TP53* mutant

reliably classify MB tumors while better predicting clinical outcome and enabling more appropriate selection of treatment options. The advancement of genomic sequencing and microarray technologies has led to the complete restructuring of the different types of MB, and this has now been incorporated into the 2016 World Health Organization (WHO) Classification of Central Nervous System Tumors [1].

MB is currently divided into five distinct molecular subtypes based on genomic alterations, gene expression profile, and response to treatment: *WNT, SHH-TP53 wild type, SHH-TP53 mutant, Group 3, and Group 4* [1, 9, 16] (Fig. 2). This novel molecular classification system has been adopted by the WHO [1], can reliably predict patient prognosis, and, in time, has the potential to drive subtype-specific treatment regimens [9, 16]. The molecular subtypes differ in their demographics, gene expression profiles, somatic genetic events, clinical outcomes, and histology (Fig. 2, Table 1) [9]. While the SHH and WNT variants are aptly named for the well-established signaling pathways that drive tumorigenesis, less is known about the genetic and molecular events driving Group 3 and 4 tumor progression. Each MB subtype will now be discussed in detail below. Demographics, genetic/molecular alterations, cell of origin, mouse models, and treatment options for each molecular subtype will be described.

Table 1 Summary of histology within the molecules MB subtypes

Molecular subtype	Histology
WNT-activated	Classic
	LCA (very rare)
SHH-activated *TP53* mutant	Classic
	LCA
	Desmoplastic/nodular (very rare)
SHH-activated *TP53* wild type	Classic
	LCA
	Desmoplastic/nodular
	Extensive nodularity
Group 3	Classic
	LCA
Group 4	Classic
	LCA (rare)

Adapted from Louis et al. [67]

WNT-Activated MB

The WNT subtype is characterized by upregulation of genes associated with the WNT signaling pathway. The prognosis of patients with WNT tumors is quite favorable, with a 5-year survival rate of 90% [9]. The 10% that do not survive in the long term often succumb to complications from therapy or secondary neoplasms caused by RT rather than WNT MB recurrence [17].

Demographics

WNT tumors represent the smallest group of MBs at just 11% of diagnoses. These tumors have a nearly equal distribution between males to females with a slight male to female dominance [9]. WNT tumors have a peak incidence at 10–12 years of age and are not typically seen in infants. Almost all tumors in this subgroup (97%) fall in the classic MB category histologically [18]. WNT tumors are typically located along the midline of the fourth ventricle and infiltrate the brainstem but only infrequently metastasize [19].

Genetic and Molecular Alterations

The first indication that mutations in the WNT pathway caused a form of MB came from the study of patients with Turcot syndrome, a rare disease that predisposes individuals to high rates of benign adenomatous polyp growths in the gastrointestinal tract and a 92-fold increased risk of developing MB [20, 21]. The WNT

signaling pathway (Fig. 3) plays a critical role in defining the midbrain-hindbrain boundary during brain development [22–24] and is important for regulating self-renewal of neural precursors during neurogenesis [25]. ß-catenin (encoded by *CTNNB1*) is the main signaling molecule in the canonical WNT signaling pathway [26, 27]. In the absence of WNT ligands, β-catenin levels are kept low in the cytoplasm. ß-catenin levels are regulated by phosphorylation-targeted destruction of β-catenin by a multi-protein destruction complex [27]. This complex is composed of the proteins Adenomatous polyposis coli (APC) and Axin, which enable the phosphorylation of β-catenin by casein kinase 1α (CK1α) and glycogen synthase kinase 3β (GSK3β) [27], leading to its eventual proteosomal destruction and subsequent gene target repression [26, 27]. Low levels of β-catenin in the nucleus also allow transcription factor T-cell-specific factor/lymphoid enhancer-binding factor (TCF/LEF) to be associated with Groucho (a gene repression cofactor) leading to target gene repression [28, 29]. However, in the presence of WNT ligands binding to frizzled (FRD) and its co-receptor LDL-receptor-related protein 5/6 (LRP), the proteosomal degradation of β-catenin is blocked resulting in an accumulation of stable β-catenin in the cytoplasm [30, 31]. In the nucleus, β-catenin displaces Groucho and activates TCF/LEF, which increases the transcription of target genes such as cyclin D1 [32, 33] and the transcription factor myelocytomatosis (*MYC*) [34, 35].

Somatic mutations of downstream WNT signaling pathway components such as *CTNNB1*, *Axin1*, and *APC* are found in sporadic MB [36–40]. The majority of WNT subtype tumors show stabilizing mutations in *CTNNB1* (70–90%) and monosomy 6 (90%) [9, 18, 41–43]. However, a small number of WNT subtype MB tumors lack mutations in *CTNNB1* and *APC*, implying that other mechanisms lead to aberrant WNT signaling and tumorigenesis in these cancers [41–44]. WNT tumors lacking *CTNNB1* mutations have been reported to exhibit mutations in the Cadherin-1 (*CDH1*) gene [41]. This protein is responsible for sequestering β-catenin at the cellular membrane, and alterations in this process may also result in aberrant activation of the WNT signaling pathway in this molecular subtype [45]. In addition to WNT signaling aberrations, approximately 50% of WNT tumors show mutations in the DEAD Box Helicase 3 (*DDX3X*) gene [41, 43]. DDX3X is a RNA helicase that has been implicated in mRNA splicing and processing, translational control, chromosome segregation, cell cycle regulation, and cancer progression [46, 47]. This is relatively specific to the WNT subtype, as only 10% of SHH and no Group 3 or 4 tumors exhibit mutations in the *DDX3X* gene [41, 43]. Other mutations such as *SMARCA4*, *CREBBP*, *TRRAP*, and *MED13*, all regulators of gene expression through chromatin remodeling, have also been discovered in WNT tumors [41, 48–51]. Aberrant WNT signaling is the dominant molecular driver of the MB subtype; however, in addition to stabilization of CTNNB1, formation of WNT MBs may require disruption of chromatin remodeling at WNT-responsive genes.

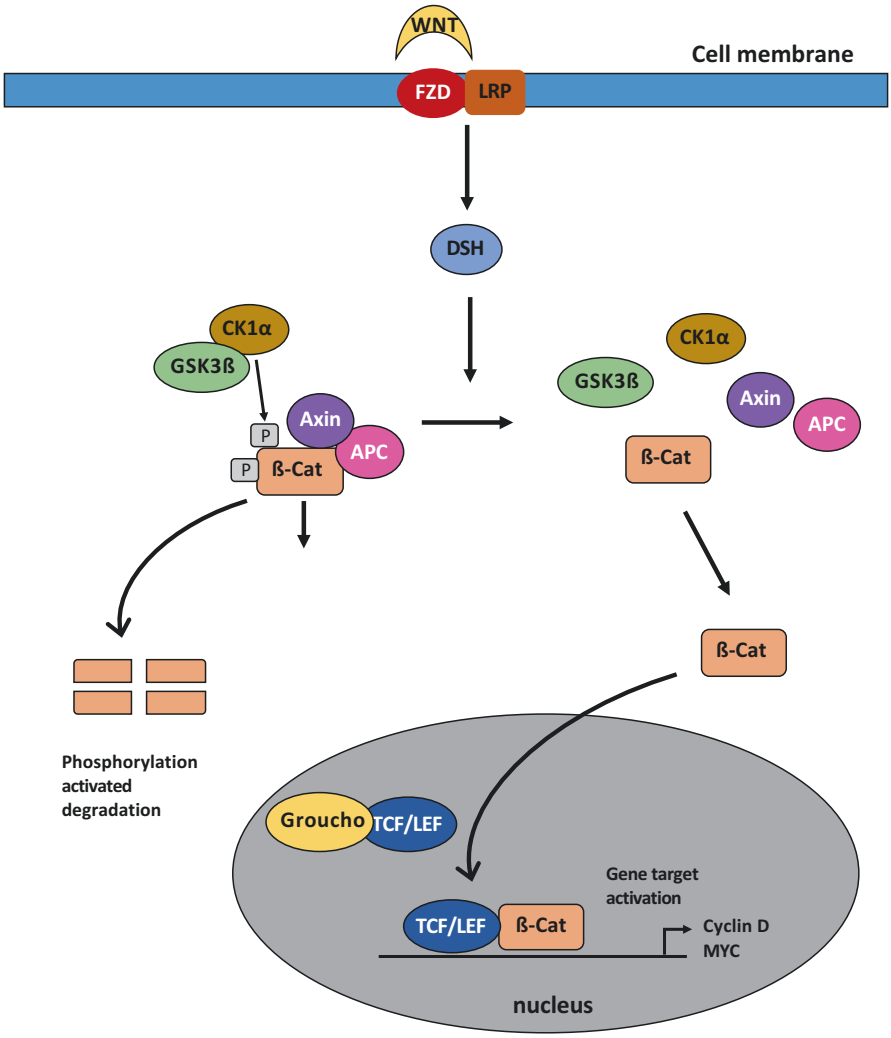

Fig. 3 The Wingless (*WNT*) pathway plays a critical role in WNT-activated medulloblastoma tumorigenesis. Canonical (β-catenin dependent) WNT signaling plays a key role in the formation of the midbrain/hindbrain boundary through the control of neural stem cell proliferation and is believed to drive tumor initiation in WNT MB. When no WNT is present, β-catenin levels are kept low through phosphorylation-targeted destruction by the multi-protein destruction complex (*Axin*, *APC*, *GSK3β*, and *CK1α*). Low levels of β-catenin leads to gene target repression. Binding of WNT to the Frizzled (*FZD*) receptor and its co-receptor LDL-receptor-related protein 5/6 (*LRP*) results in elevated intracellular β-catenin through the inhibition of targeted destruction. Stable non-phosphorylated β-catenin is translocated into the nucleus where it displaces Groucho and activates TCF/LEF, enabling transcription of target genes such as cyclin D1 and MYC

Cell of Origin and Mouse Models of WNT-Activated MB

Tumors of the WNT subtype have been shown to arise from lower rhombic lip progenitor cells of the dorsal brainstem [52]. An activating mutation in *Ctnnb1* disrupts the normal differentiation and migration of these progenitor cells resulting in abnormal accumulation of cells [52]. Regional expression of 24 WNT MB signature genes was charted using software that generates three-dimensional gene expression maps across the developing mouse brain [52]. WNT MB signature genes were predominately expressed in the lower rhombic lip at embryonic day (E) 11.5 and in the dorsal brainstem at E15.5 [52]. Based on this data, mice were generated that carry *Ctnnb1* mutations in the progenitor populations of the hindbrain. *Ctnnb1* mutations were selectively expressed in cells that exclusively express the *Blbp* gene, which includes the ventricular zone progenitors. *Ctnnb1* mutations were also expressed only in granule neural precursor cells (GNPCs) using the enhancer of the *Atoh*/*Math1* gene. No persistent cellular masses or tumors were found in the cerebellum or dorsal brainstem of mice harboring the *Ctnnb1* tumors in GNPCs [52], while *Blbp*-driven *Ctnnb1* mutations in mice formed aberrant cell collections in the dorsal brainstem. However, in the *Blbp*-driven *Ctnnb1*mice, only animals that harbored an additional mutation in the *TP53* gene formed classic medulloblastoma that were confined to the dorsal brainstem and displayed expression profiles similar to WNT-subtype MB [52]. Together, these studies show that aberrant WNT signaling in the progenitor cells of the dorsal brainstem gives rise to WNT MB, providing the first evidence for the cell of origin for this subtype.

Treatment

Patients with WNT MB undergo standard current treatment including surgery, chemotherapy, and RT. However, as WNT tumors exhibit a relatively good prognosis, it is recommended that patients with nonmetastatic disease receive reduced chemotherapy and RT [6]. Although there are small-molecule inhibitors of the WNT signaling pathway, crosstalk between signaling pathways necessitates treatment options that can target multiple pathways [53]. For example, the WNT and PI3K/AKT signaling pathways have been shown to exhibit crosstalk; thus small-molecule inhibitors targeting PI3K/AKT have been suggested to inhibit WNT signaling. Baryawno et al. [54] have shown that small-molecule PI3K/AKT inhibitors such as OSU03012 decrease WNT signaling by activating GSK-3 β and promoting degradation of β-catenin Recently, the anticancer compound norcantharidin (NCTD) has also been shown to impair nuclear translocation of β-catenin signaling and reduces tumor growth in an orthotopic mouse xenograft model of MB [55].

SHH-Activated MB

Demographics

The SHH subtype is characterized by upregulation of genes associated with the SHH signaling pathway. Similar to WNT tumors, SHH tumors occur in a 1:1 ratio in males/females, with SHH tumors found predominantly in infants and adults and rarely in children aged 3–16 [9, 16]. These tumors make up approximately 28% of all MBs diagnosed and have an intermediate prognosis [9]. SHH MBs frequently arise in a cerebellar hemisphere in adults [52] and in the cerebellar vermis in children [16]. The prognosis of SHH tumors differs significantly between age groups. The 5- and 10-year overall survival (OS) rate in infants is 77%, which drops to 68% and 51% 5- and 10-year OS, respectively, in children and 75% and 34% 5- and 10-year OS, respectively, in adults [18]. This difference in survival between age groups is most likely attributed to the high percent of SHH tumors in infants exhibiting desmoplastic/extensive nodularity which has been shown to be a positive prognostic factor in these young patients [56]. Nearly all desmoplastic/nodular variants are of the SHH subtype; however, 50% of all SHH tumors are not desmoplastic [9], highlighting the importance of finding alternatives to histopathological diagnosis and the need for widespread clinical acceptance of the molecular subtype classification system.

Genetic and Molecular Alterations

The SHH signaling pathway (Fig. 4) plays an essential role in the control of GNPC proliferation in the external granular layer (EGL), as well as glial differentiation in the cerebellar cortex [57–59], and is believed to drive tumor initiation in the SHH MB subgroup [9]. The membrane-bound receptor Patched (PTCH) plays an inhibitory role that represses SHH signaling when it is unbound [60]. Binding of the SHH ligand to PTCH releases the inhibitory effect PTCH has on Smoothened (SMO), a member of the G protein-coupled receptor family [61]. De-inhibition of SMO results in the activation of the zinc-finger proteins of the glioma-associated oncogene (GLI) transcription factor family including GLI1, GLI2, and GLI3 [60]. GLI proteins can function as either transcription activators or repressors. In the absence of SHH, GLI2 and GLI3 are phosphorylated leading to their proteolytic cleavage to generate their repressor forms [60]. With the activation of SMO, transcriptionally active forms of GLI are formed in combination with inhibition of suppressor of fused (SUFU), a protein responsible for sequestering GLI in the cytoplasm [60, 62]. Inhibition of SUFU allows the activating forms of GLI to translocate to the nucleus where they replace the repressor forms of GLI on target genes leading to transcriptional activation [62].

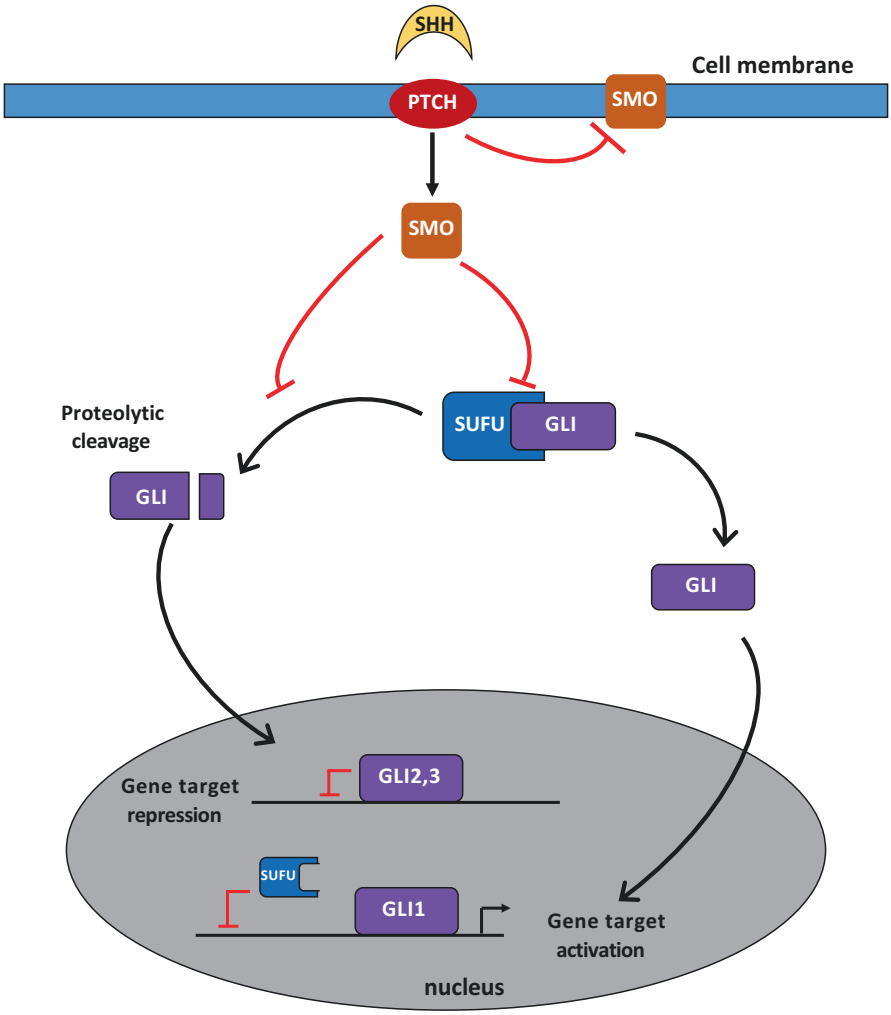

Fig. 4 Sonic hedgehog (*SHH*) signaling pathway plays a critical role in SHH-activated medulloblastoma tumorigenesis. The SHH signaling pathway plays an essential role in the control of GNPC proliferation in the EGL and is believed to drive tumor initiation in SHH MB. When SHH is not present, the Patched (*PTCH*) receptor plays an inhibitory role repressing SHH signaling. In the absence of SHH, GLI2 and GLI3 are phosphorylated leading to proteolytic cleavage to generate their repressor forms. Binding of SHH to PTCH releases the inhibitory effect PTCH has on Smoothened (*SMO*). De-inhibition of SMO results in activation of GLI transcription factors. Suppressor of fused (*SUFU*) is found in the cytoplasm and nucleus and plays a role in sequestering GLI proteins when SHH is not bound to PTCH. Binding of SHH to PTCH leads to inhibition of SUFU resulting in translocation of GLI to the nucleus. SHH binding ultimately results in targeted gene activation through a complex signaling pathway

The link between MB and the SHH pathway was made through studies of individuals with Gorlin syndrome. Gorlin syndrome (also known as nevoid basal cell carcinoma syndrome) is a disease that results from hereditary mutations in the SHH receptor *PTCH.* Gorlin syndrome is characterized by macrocephaly, skeletal abnormalities, and, in some patients, a high rate of cancer, including basal cell carcinomas and MB [9, 63]. Germline mutations of the SHH inhibitor *SUFU* also predispose individuals to MB. In addition, somatic mutations of *PTCH, SMO*, and *SUFU,* as well as amplification of *GLI1* and *GLI2,* have been found in sporadic MB, pointing toward the SHH signaling pathway as the primary driver of tumorigenesis in this MB subtype. Deletion of chromosome 9q, the location of the *PTCH* gene, is also limited to SHH MB and is the most common chromosomal abnormality found in this subtype [16, 64, 65]. Other genomic abnormalities include gain of chromosomes 3q and 9p and loss of 10q, 20p, and 21p [64, 66].

Heterogeneity at the genetic and molecular level has led to recent discovery of additional subtypes within SHH-activated MBs [67]. In a large cohort study, it was shown that mutations in *TP53* are found in 21% of SHH MB tumors, and this was found almost exclusively in patients between 5 and 18, a rare age for this molecular variant. In addition, 72% of patients aged 5 or older who succumbed to their disease had harbored a *TP53* mutation [68]. *TP53* mutation status was shown to be the most important independent risk factor in SHH-variant MB when compared to age, sex, histology, and presence of metastasis at diagnosis [68]. The prognostic value of *TP53* mutation status has since allowed subclassification of these tumors into "SHH-activated *TP53*-wild type" or "SHH-activated *TP53* mutant" subgroups (Fig. 3). *TP53*-wild-type patients have a 5-year OS of 81%, whereas patients with *TP53* mutations exhibit a 41% 5-year OS. This new SHH subtyping has been integrated into the 2016 WHO classification of CNS tumors [1].

Cell of Origin and Mouse Models of SHH-Activated MB

During normal development of the cerebellum, SHH signaling plays a crucial role in the proliferation of GNPCs in the EGL. As SHH signaling is decreased, GNPCs begin to differentiate and migrate inward to the internal granule layer (IGL). Aberrant SHH signaling may result in prolonged proliferation of GNPCs in the EGL, the anatomical region where SHH tumors originate. Schüller et al. [69] demonstrated that both early multipotent progenitors (GFAP+ and Olig2+) and late unipotent (Atoh1+/Math1+) progenitor cells of the cerebellum can give rise to SHH MB. However, the acquisition of GNPC identity is crucial for SHH MB tumorigenesis. Similarly, Yang et al. [70] showed that deletion of *Ptch* and overactivation of the SHH pathway can result in MB in both neural stem cells (GFAP+) and GNPCs (Atoh1+/Math1+), but only after commitment to, and expansion of, the neuronal

lineage. These studies show that SHH MB can develop from neural stem cells or GNPC progenitor cells of the cerebellum; however, commitment to the GNPC lineage is a necessary step in SHH MB tumorigenesis, providing evidence that GNPCs are the cell of origin for SHH MB [69, 70].

Many transgenic and knockout mouse models have been generated to study SHH MB initiation and progression. Since dysregulation of the SHH signaling pathway is a major contributor to SHH MB tumorigenesis, mouse models are typically generated by genetic manipulations of SHH pathway genes. Deletion of *PTCH1* or *SUFU* as well as activation of *SMO* in mice results in tumors that resemble human SHH MB [71–73].

Treatment

Activation of SHH signaling pathway in SHH MBs has been extensively studied. Accordingly, SMO inhibitors such as cyclopamine and vismodegib are being evaluated in clinical trials for MB patients with SHH pathway activation [74]. Cyclopamine is a naturally occurring small-molecule inhibitor that suppresses SHH signaling by binding to SMO [75, 76]. However, the potency of cyclopamine is relatively low; thus synthetic SMO inhibitors such as vismodegib, sonidegib, and saridegib have also been utilized [77–80]. Although SMO inhibitors are initially successful, patients eventually relapse due to drug resistance. This is partially attributed to mutations of downstream targets such as SUFU and activation of GLI in the absence of SMO [74, 81, 82]. Furthermore, patients with *TP53* mutations are resistant to vismodegib treatment [83]. Importantly, Morrissy et al. have recently shown that genetic events in a murine model of recurrent SHH MB exhibit poor overlap with the matched primary tumors [84]. Whole genome sequencing in human samples also demonstrated genetic divergence between matched tumors at diagnosis and post-therapy [84]. Thus, targeted therapy against the primary tumor will most likely be ineffective against recurrent disease resulting in failed clinical trials and no significant benefit to the patient.

As SHH tumors also exhibit upregulation of other signaling pathways such as NOTCH and PI3K, crosstalk between these pathways may play a role in treatment resistance. There is conflicting evidence regarding the importance of the NOTCH pathway in SHH-activated MBs. Whereas Hallahan et al. demonstrated that targeting the NOTCH pathway with ϒ-secretase inhibitors decreases proliferation and increases apoptosis in a SHH MB xenograft model [71], Hatton et al. have shown that targeting the NOTCH pathway is not beneficial in SHH MBs [85]. However, several studies have shown that genes associated with PI3K pathway contribute to SHH MB drug resistance [86–88]. In addition, PI3K pathway inhibitors in combination with SHH pathway inhibitors have demonstrated enhanced efficacy and improve survival in MB orthotopic xenograft mouse models [86, 89]. Therefore, a combinatorial treatment approach will be necessary to treat SHH tumors.

Group 3 and Group 4 MB

These two "non-SHH/WNT" subtypes share some similar clinical presentations and molecular characteristics and will therefore be discussed together. Of the MB subtypes, the least is known about Groups 3 and 4.

Demographics

Group 3 tumors are found 2:1 in males compared to females and are found predominantly in infants and children, but rarely in adults [9]. Group 3 makes up approximately 27% of MB diagnoses [65]. The majority of Group 3 tumors are classic MB histology, with some desmoplastic and LCA cases [9]. Group 3 patients have the worst prognosis of the four subtypes, with infants having a 5-year OS of 45% and 10-year OS of 39%, while children have a 5- and 10-year OS of 58% and 50%, respectively [65]. Group 3 tumors have a very high rate of metastasis, which is a major contributor to their poor prognosis.

Group 4 makes up the most common subtype of MB (34%) [65]. However, this molecular variant is also the least understood [9]. Similar to Group 3, most Group 4 MBs exhibit classic histology, with some desmoplastic and LCA cases [9]. Gender distribution is approximately 2:1 males to females, with this subtype arising across all age groups, peaking in children [16]. Group 4 tumors have an intermediate prognosis, similar to the SHH subtype [9].

Genetic and Molecular Alterations

As opposed to the WNT and SHH subgroups, less is known about the molecular drivers of Group 3 and Group 4 variants. Evaluation of genetic abnormalities and gene expression has revealed that Group 3 is the only variant associated with amplification and overexpression of *MYC* but not *MYCN* [9, 43]. While WNT tumors exhibit *MYC* amplifications, they also show amplifications in *MYCN* [16, 43]. Further subgrouping within Group 3 has been proposed based on the presence of *MYC* amplification. Subset Group 3α (15%), those patients with *MYC* amplification, are at a much higher risk of recurrence and death when compared to Group 3β patients with no *MYC* amplification.

Isochromosome 17q is the most common cytogenetic change observed in Group 3 and 4 tumors, occurring in 26% of all Group 3 tumors and 66% of Group 4 tumors [16, 43]. Other cytogenetic changes seen in Group 3 and 4 tumors include 17p deletion, gain of chromosome 1q, and loss of chromosomes 5q and 10q. Group 3 tumors are more likely to show gain of chromosome 1q and/or loss of chromosomes 5q and 10q [9]. Disruptions of chromatin genes that are associated with histone methylation

have also been found in MB. These epigenetic disruptions are likely subtype specific and are necessary components of MB tumorigenesis [41, 43, 48–51]. Mutations in genes including *EZH2, KDM6A, CHD7*, and *ZMYM3* may disrupt chromatin marking of genes such as *OTX2*, *MYC*, and *MYCN* in Group 3 and 4 tumors [41, 43].

To date, no definitive genetic events or transcriptional pathways have been shown to drive tumorigenesis in Group 4. Group 4 tumors have little to no expression of MYC or MYCN except for a few tumors that show *MYCN* amplification [16, 43]. However, the oncogenes and developmental transcription factors *OTX2* and *FOXG1B* are amplified and overexpressed in both Group 3 and 4 tumors [16]. Interestingly, it has recently been shown that *OTX2* regulates MB stem cell function in a subgroup-dependent manner. While *OTX2* plays an inhibitory role when overexpressed in SHH MB, it is oncogenic in Group 3 and 4 MBs by promoting growth and self-renewal or "stemness" while inhibiting differentiation in vitro and increasing tumor growth from MB stem cells in vivo [90]. Further evaluation of the mechanisms regulated by OTX2 in Group 3 and 4 MBs will provide a better understanding of the molecular signatures that contribute to pathogenesis of these highly aggressive subtypes.

It is unclear why Group 3 and 4 tumors have a higher propensity to occur in males compared to females. This may be partially explained by the recurrently mutated genes, *ZMYM3* and *KDM6A*, which are located on the X chromosome [41]. *ZMYM3* and *KDM6A* mutations are found almost exclusively in tumors from males [41, 91]. In addition, 80% of all females with Group 4 tumors show a loss of the X chromosome within this MB subtype [9].

Cell of Origin and Mouse Models of Group 3 and 4 MBs

Although less is known about the cell of origin for Group 3 and 4 tumors, two independent groups developed a mouse model that recapitulated Group 3 Myc-subtype tumors [92, 93]. Overexpression of MYC combined with *TP53* mutation resulted in highly aggressive tumors that histologically and molecularly resemble Group 3 tumors, albeit using different cells of origin. While Kawauchi et al. [92] overexpressed MYC in GNPCs, Pei et al. [93] used cerebellar stem cells (Prominin1/CD133+, Lineage-), both of which resulted in similar tumor phenotypes. Expression profiles showed that both Myc-driven tumors exhibit significant similarities to neural stem cells, induced pluripotent stem cells, and embryonic stem cells, suggesting that Group 3 MB may arise from a neural stem cell or a dedifferentiated GNPC [92–94].

There is currently no reliable mouse model of Group 4 MB, making it difficult to pinpoint the cell of origin. However, it has been recently proposed that Group 4 MBs arise from progenitor cells of the upper rhombic lip [95]. Lin et al. have shown that three master regulator transcription factors (LMX1A, EOMES, and LHX2) in Group 4 tumors exhibit overlapping spatiotemporal expression in deep cerebellar nuclei of the nuclear transitory zone [95]. These studies suggest that deep cerebellar

nuclei, or their earlier precursors from the upper rhombic lip, are the putative cell of origin for Group 4 tumors.

Treatment

Group 3 MBs are the most aggressive subtype of MBs and exhibit frequent metastasis, resulting in difficulties treating these tumors with standard treatment options. Thus, there is a critical need to identify the pathways contributing to Group 3 MB pathogenesis not only to better understand how these tumors progress but also to develop targeted therapies with less harmful side effects on the developing brains of children. Although little is known about the driver mutations in Group 3 tumors, *MYC* amplification in these tumors provides a target for Group 3 MB treatment. For example, Morfouace et al. [96] have identified two FDA-approved compounds, pemetrexed and gemcitabine, that preferentially inhibit proliferation of Group 3 tumors that exhibit *MYC* amplification or overexpression. Moreover, the combination of these two drugs results in an increased survival in a Group 3 mouse xenograft model [96]. The lack of a preclinical model to study Group 4 MBs has hampered the development of targeted therapy for these tumors. However, since OTX2 is amplified or overexpressed in both Group 3 and 4 MBs, this transcription factor and/or its downstream effectors provide potential therapeutic targets for these subtypes. Although there is currently no treatment targeting OTX2 specifically, studies have shown that the use of retinoic acid can reduce OTX2 expression and induces neuronal differentiation [97]. However, tumor cells quickly become resistance to retinoic acid treatment, and different MB cell lines exhibit variable responses [98, 99]. In order to develop novel targeted therapeutics for Group 3 and 4 MBs, a much better understanding of the underlying mechanisms associated with tumor progression and metastasis is required.

Diagnostic Imaging in the Molecular Era

The typical MB appears as a well-defined, homogeneous tumor localized within the vermis, with marked contrast enhancement on preoperative computed tomography (CT) scanning [100]. On magnetic resonance imaging (MRI), tumors are hypointense on T1 and hyperintense on T2-weighted images and show marked contrast enhancement (Fig. 5). The number of patients with an atypical phenotype is low.

Recently, Perreault et al. [101] demonstrated that tumor location and enhancement patterns were correlated with specific MB subtypes suggesting that MRI may potentially serve as a complement to genomic diagnostic testing for these tumors. Seventy-five percent of WNT tumors occurred uniquely along the cerebellar pontine and the cerebellar pontine angle (CP/CPA); however, these data conflict with previous studies that demonstrated midline occurrence [102] or midline occurrence

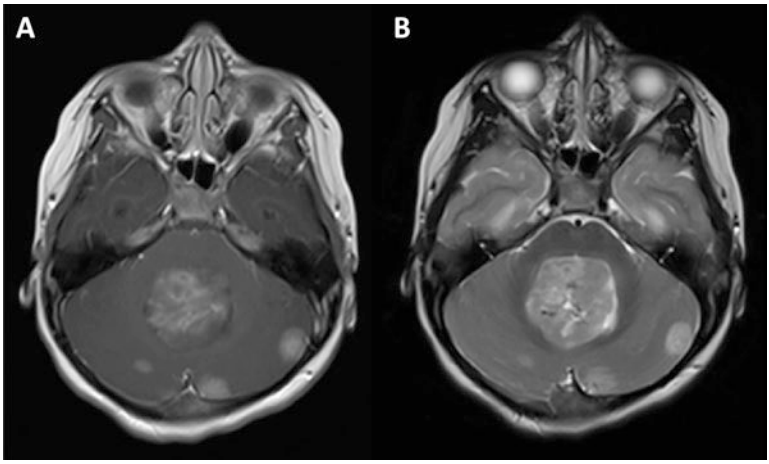

Fig. 5 Five year old with multifocal medulloblastoma with a heterogeneous mass in the fourth ventricle and several other enhancing lesions in the posterior fossa. (**a**) T1 post-gadolinium image. (**b**) T2-weighted image

concomitant with dorsal brainstem infiltration [52]. The majority (54%) of SHH tumors were located in the cerebellar hemispheres, and this result was consistent with previously reported findings [102]. In contrast, Groups 3 and 4 MBs were primarily midline and occupied the fourth ventricle. Interestingly, tumor margins were not well defined in Group 3 MBs, and in Group 4 tumors, very minimal or no enhancement was observed [101]. This characteristic distinguished Group 4 from Group 3 MBs and may prove useful for differential diagnosis.

The other MRI features including cysts, peritumoral edema, and tumoral necrosis were not characteristic of specific molecular subgroups. Diffusion-weighted imaging (DWI) did not significantly differ among the molecular subgroups [101]. Collectively, these results suggested that using MRI to predict MB molecular subgroups might have additional diagnostic value in centers where genetic/molecular testing is limited.

Cancer Stem Cells and Their Contribution to MB Tumor Heterogeneity

Characterization of the extensive genetic and molecular heterogeneity in MB has led to the current classification system [1]. However, there are additional layers of heterogeneity to consider, including the cancer stem cell hierarchy.

The cancer stem cell (CSC) theory has been employed to explain the cellular and functional heterogeneity found between and within tumor subtypes, including MB. CSCs are a subpopulation of tumor cells that exhibit stem cell-like properties

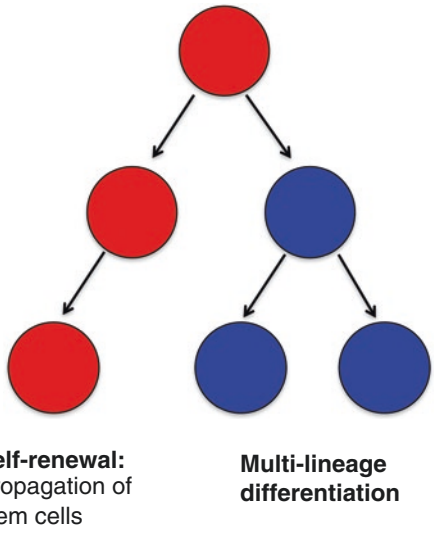

Fig. 6 Stem cells and cancer stem cells possess two characteristics, self-renewal and multi-lineage differentiation. Self-renewal is the ability to propagate oneself indefinitely and is a defining stem cell feature. Progenitors cells exhibit limited self-renewal capacity and ultimately differentiate. Self-renewal can occur as an asymmetrical division, whereby one stem cell gives rise to one stem cell and one further differentiated cell. In asymmetrical stem cell division, the stem cell population does not expand and is therefore maintained through subsequent cell divisions. Alternatively, stem cells can undergo symmetrical division, whereby one stem cell gives rise to two stem cells (not shown). This allows for exponential expansion of the stem cell population. *Red* denotes a stem cell; *blue* denotes a further differentiated cell such as a transit-amplifying progenitor cell

including self-renewal capacity and multi-lineage differentiation, and are responsible for tumor initiation and maintenance (Fig. 6) [103]. The differentiation of CSCs provides a mechanism for the generation of phenotypic and functional heterogeneity that cannot be fully attributed to clonal evolution and environmental pressures. Singh et al. [104] first demonstrated that brain tumors contain CSCs, also known as brain tumor-propagating cells (BTPCs). They further identified a cell surface marker CD133 (Prominin1), which selects for a highly self-renewing cell population in both MB and glioblastoma [104]. While CD133 is the most commonly utilized BTPC marker, little is known about its biological function. Read et al. and Ward et al. both identified an additional BTPC marker, CD15/SSEA1 (Stage-specific antigen 1) in a *Ptch* mutant mouse model of SHH MB [105, 106]. While Read et al. [105] demonstrated that Math1+/CD15+ neuronal progenitors are responsible for tumor propagation, Ward et al. [106] suggested that CD15 selects for a stem cell population rather than progenitor cells. More recently, both Ahlfeld et al. and Vanner et al. demonstrated that the stem cell marker Sox2 also plays a role in SHH MB tumor propagation [107, 108]. These authors showed that following treatment with chemotherapy and SHH pathway antagonists, the $Sox2^{+}$ cell population was enriched resulting in tumor growth and relapse [107]. Although Ward et al. and

Read et al. demonstrated that CD15 can be used to isolate BTPCs in *Ptch*-driven mouse models of SHH MB [105, 106], further work by Vanner et al. has shown that in order to reliably isolate the BTPC population, CD15 must be used in combination with Sox2. Recent studies have also identified a role for the transmembrane neurotrophin receptor, CD271 (p75NTR), in regulating stem/progenitor cells in SHH MB [109]. Liang et al. have shown that CD271 expression is mostly associated with SHH MB, and functional characterization revealed a role for this cell surface marker in SHH MB tumor propagation and maintenance through modulating stem cell properties [109]. Identification and functional characterization of additional cell surface markers that select for BTPC populations will provide further insight into the cellular complexity within these tumors.

The Future of Medulloblastoma Research

The extensive heterogeneity between and within the MB subtypes is just beginning to be unraveled. With advancement in technologies and identification of novel MB biomarkers, further dissection of the heterogeneity within the current molecular subgroups may lead to discovery of additional subtypes. Yet, current standard treatments still do not take into account the molecular variants. Consideration of the MB subtypes will be imperative to improve diagnosis and allow for selection of the most appropriate treatment regimens. Identification and functional characterization of subtype-specific biomarkers will ultimately lead to the development of novel diagnostic tools and targeted therapies that will improve the quality of life for the increasing number of children who survive these devastating tumors.

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Can Cerebellar Neurodevelopmental Disorders Affect Behavioral Disorders or Vice Versa?

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Abstract Recent investigations have been focused on understanding the role of the cerebellum in non-motor behaviors and of the cerebellar dysfunction in neurodevelopmental, neurobehavioral, and schizo-affective disorders. Non-motor behaviors, including emotion, cognition, and social behavior, seem to be modified by impairment of the cerebellar structure-function relationship. Clinically, these behavioral defects have been observed in patients with autism spectrum disorders (ASD), attention deficit hyperactivity disorder (ADHD), and schizophrenia. These behavioral outcomes have been demonstrated to be associated with prenatal and/or early postnatal damages of cerebro-cerebellar circuits. Concerning to the essential role of the cerebellum in early neurodevelopment, understanding the association between cerebellar injury and long-term alteration in behavior is highly crucial. This chapter's attempts are to summarize the recent evidence of involvement of the cerebellum in neurodevelopment and behavior and that both these views remain to be revised for declaration of the paradoxical relationship between cerebellar function and behavioral despair, as well as neurodevelopmental disorders including ASD and ADHD.

Keywords Cerebellum • Neurodevelopment • Behavioral despair • Schizo-affective disorders

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Introduction

The cerebellum is traditionally considered as the brain region that is involved in the motor and non-motor activities [30, 83]. Regarding to the major role of the cerebellum in the posture and movements, preliminary studies showed that elimination of this area can be resulted in disruption of these activities [40]. These were in line with clinical reports demonstrating that the cerebellum degeneration may impair posture and speech, voluntary movement of extremities, and gait [42]. Several studies have been performed to understand the exact function of the cerebellum [43] and to find the importance of this area in control of motor movements [85] and learning [45]. On the other hand, the evidences have shown that extensive connections of the cerebellum with the other brain regions (e.g., prefrontal and posterior parietal cortex) are associated with non-motor tasks [16, 17]. Lately, imaging techniques have demonstrated an association between function of the cerebellum with cognitive processes such as language [74], attention [2], and affective processes [38]. Therefore, it is believed that alteration of cerebellar structure and function can be concluded to several abnormalities in the emotional, cognitive, and social domains which were observed in patients with such neurodevelopmental disorders as autism spectrum disorders (ASD) and behavioral despair [61, 62, 79]. In accordance to the complex neurobiology of neurodevelopmental disorders and behavioral despair, the role of the cerebellum in the non-motor functions should be well defined [7].

In this chapter, we provide a brief summary of the importance of the cerebellum in pathophysiology of neurodevelopmental and behavioral disorders. Although the cerebellum has been found to be involved in neurodevelopmental disorders, structural and functional differences in different regions of the cerebellum play a role in attention deficit hyperactivity disorder (ADHD), developmental dyslexia, and ASD. This suggests the hypothesis that involvement of different cerebro-cerebellar circuits may result in the differences between the neurodevelopmental disorders [86]. In addition to these disorders, there are such neurodevelopmental disorders as developmental coordination disorder (DCD), which frequently co-occur with the abovementioned neurodevelopmental disorders (e.g., ADHD and dyslexia) hypothesizing a relation to cerebellar dysfunction [10, 109]. This information makes a question in mind how the cerebellar dysfunction affects developmental processes and causes developmental disorders and differences in localization of cerebellar dysfunction may cause different disorders.

The cerebellar growth has been enormously occurred during the first 24–40 weeks of pregnancy, leading to approximately fivefold in volume and over 30-fold in surface area [18, 102]. The cerebellar growth continues throughout the first postnatal year, although the neural differentiation and growth of axonal inputs and outputs occur slower than prenatal stage [18, 102]. The process can interpret this event that premature infants are encountered an increased risk of cerebellar developmental disorder, hemorrhages, and future neurodevelopmental disabilities [18, 102]. As a result, cerebellar injury in childhood may lead to a range of long-term motor, cognitive, and affective disorders with poorer outcomes than cerebellar damage in

adulthood [82, 103]. The findings put the cerebellum at center point of neurological investigations of neurodevelopmental disorders, such as ASD [18, 31]. The confidential evidences emphasized to an obvious association between the cerebral cortex damage at early life result in an elevated risk of affective and attention deficits, internalizing behavioral disorders, and withdrawal from social contact [64, 103]. Consistent with cerebellar tumor and/or resection of the tumor in children, an abnormally increased risk of cognitive and adaptive impairments [9], as well as the vermis injury, has been shown to be related to long-term affective dysregulation [63]. Also, it is demonstrated that the vermis malformations are involved in higher rates of affective and behavioral disorders, including ASD [18, 92]. Congenital cerebellar malformations, as well as a variety of early cerebellar lesions, have a direct relation to ASD. To conclude these findings, some scientists like Schmahmann et al. included ASD among the category of psychiatric disorders associated with cerebellar damage or disease [81]. The studies have demonstrated that cerebellar injury in infancy is one of the major risk factors, which increases approximately 40-fold in developing ASD [64, 103]. The evaluation of different pathological conditions damaging the cerebellum has verified the relationship between the injuries and ASD. For instance, tuber load in the cerebellum in children with tuberous sclerosis is considered as a specific predictor of ASD [18, 104]. The cerebellar damage may cause some complications, including gaze aversion, stereotyped movements, linguistic impairments, as well as complete avoidance of physical contact, ultimately leading to ASD [78]. In line with the basic and experimental findings, the clinical evidences have been suggested that cerebellar injury at early stages via developmental diaschisis can affect the development of cerebral cortical area to which the cerebellum projects [103]. Therefore, not only cerebellar function but also the structure and function of multiple regions of the cerebral cortex can negatively be influenced by cerebellar developmental differences in patients with ASD.

Several investigations of patients with ASD have indicated to an abnormal modification of size and shape of neurons of the deep cerebellar nuclei, as well as decrement of the number of Purkinje cells (PC) [4, 51, 52, 70, 83]. Postmortem studies have confirmed the experimental results showing a reduction in gyrification, and in size of granular and molecular layers of the vermis, along with loss of PC [7, 67, 91]. These findings may adjust the hypothesis that ASD has a prenatal origin of defects, which continue at early postnatal stage [31]. Neuroimaging techniques, such as structural magnetic resonance imaging (MRI), have presented conflicting information that vermal hypoplasia is observed in the majority of individuals with ASD. To confirm these data, studies using functional MRI have further implied that patients with ASD exhibit abnormal function of the cerebellum ([111, [31, 100]). The neuroimaging studies also have shown an alteration in anatomical and functional connectivity of the cerebellum with other regions of the brain, including the thalamus and cerebral cortex [66, 83, 101].

In addition to the neuroimaging studies, pharmacological investigations have demonstrated that the cerebellar glutaminergic and GABAergic systems are considered as a target of dysfunction in ASD patients [11, 83].

Also, major psychiatric disorders, including major depressive and bipolar disorders and schizophrenia, are hypothesized to be contributed by comprehensive alteration in GABAergic signaling system, such as changed expression of cerebellar GABA receptor [83]. This can be associated with reduction in expression of FMRP and alteration in FMRP-mGluR5 signaling and its downstream targets including RAC1, APP, STEP, and homer 1. On the other hand, expression of GABA receptor is influenced by epigenetics or monoallelic expression. As a result, agonism of GABAergic receptors, modulation of mGluR5 activity, and inhibition of glutamate-induced excitotoxicity that may be potential therapeutic strategies, along with the drugs, affect monoamine systems including dopaminergic or serotonergic pathways [32–34]. Actually, GABAergic system can be an important target for novel medication for the psychiatric disorders [36].

Furthermore, the literature resulted from gene and protein expression analyses have demonstrated the downregulation of synaptophisin, SNAP-25 (synaptosome-associated protein), and complexin, as well as upregulation of semaphorin 3A, an axonal chemorepellant [28, 29, 68, 83]. Interestingly, a dysregulation of activity and levels of D-amino acid oxidase (DAO), the enzyme that metabolizes D-serine, a co-agonist of NMDA (N-methyl-D-aspartate) receptor, was also observed [14]. Therefore, the available evidence seem to indicate to disease-specific, including decreased volume of the vermis, and nonspecific pathological factors, including reduction in the number PC and pharmacological changes of the cerebellum in the neurodevelopmental disorders [83].

In addition to ASD, the cerebellum has been suggested to be involved in schizophrenia, demonstrating coordination and postural abnormalities, impaired eyeblink conditioning, and procedural learning deficits [53, 54, 84]. The neurological signs are thought to be related to structural alterations in the cerebellum [7, 106]. Regarding to the extensive connections between the cerebellum and forebrain regions, cognitive dysmetria and poor mental coordination are proposed to be produced by cerebellar abnormalities in schizophrenic patients [5, 6].

Contribution of the Cerebellum in Neurodevelopment

There are increasing evidences emphasizing the major role of the cerebellum in the development of the brain. The studies of fetal, neonatal, and pediatric individuals support the hypothesis that the developing cerebellum clearly participates in motor, cognitive, and socio-behavioral development and exerts the role that is associated with a regional functional topography of the cerebellum. Consistent with these data, investigational studies have indicated to the relationship between early life and older children with cerebellar injury (e.g., pediatric posterior fossa tumors), and infants with cerebellar malformations and neurodevelopmental disorders, clarifying the importance of cerebellar structure-function relationships in the brain development [87].

The developmental process of the cerebellum possesses a highly regulated pattern, as which more rapidly grows during 20–40 weeks of gestation in comparison with other cerebral structure, demonstrating the importance of the critical period for cerebellar development [15, 65]. Thus, the vulnerability of the cerebellum and its developmental repercussions of injury can disrupt this highly orchestrated, programmed developmental process during the important period. On the other hand, disruption of cerebellar growth significantly affects other regions of the brain, for instance, the developing cerebral cortex [103]. This is related to the richly interconnection of the cerebellum with different areas of the cerebral cortex supporting movement, cognition, and affective regulation [89]. In this regard, the cerebellum seems to play a modulatory role in the cerebro-cerebellar circuits, supporting the behavioral optimization, particularly in procedural learning and skill acquisition [87].

Subsequently, it is believed that early disruption of the cerebellum, due to prenatal cerebellar developmental lesions (i.e., malformations), preterm birth, and cerebellar posterior fossa tumors in early childhood, can lead to neurodevelopmental disorders with long-lasting and wide-ranging alterations in the structure and function of cerebro-cerebellar systems that result in long-term behavioral disorders [87].

Role of the Cerebellum in Adaptive Behaviors, Autism Spectrum, and Neuropsychiatric Disorders

It is evident that cerebellar tumor removal in children and cerebellar parenchymal injury in very preterm infants resulted in impairment of adaptive behaviors [9] and a variety of affective disorders [63, 64]. For instance, affective dysregulation is associated with cerebellar dysfunction in children [63], while emotional lability is also observed following posterior fossa syndrome [75].

Regarding to specific structure-function relationship, an association between the posterior vermis injury and vermal lesions with behavioral dysregulation, flattened affect, and disinhibited behavior was observed [1, 21, 63]. Some reports have mentioned that most of the children with midline or vermal tumors are encountered to affect dysregulation [1]. These findings were supported by a study by Richter et al. [77] that both positive (e.g., decreased aggression and thoughtful behavior) and negative (e.g., depression, anxiety, and aggression) behavioral symptoms were seen in children with chronic cerebellar lesions. The association between the vermis and behavioral regulation pays our attention to the important role of the posterior vermis and its defects in neurodevelopmental disorders, including ADHD [48] and autism [8].

In addition, Schmahmann implied that more than half of the surviving preterm infants with damage of parenchymal tissue of the cerebellum show psychiatric disorders [81] and functional limitations in socialization skills. Also, distinct socio-behavioral defects of attention, affective, internalizing, and pervasive subdomains were reported in the children with cerebellar injury [64].

Taken together, the reports have shown that cerebellar injury and lesion at early life in preterm infants are associated with wide-ranging neurodevelopmental disorders [13]. Moreover, a reduction in volume of the posterior vermis is thought to be in line with neurodevelopmental-related behavioral dysregulation, including autism and ADHD. Psychiatric disorders are also reported to be correlated with cerebellar injury during childhood [87].

Cerebellum Plays a Role in ASD

Evidences have proposed that dysfunction in specific regions of the cerebellum can result in neurodevelopmental disorders, including ASD, according to the involvement of the cerebellum in the developing brain. Scientists have demonstrated the major role of cerebellar damage in the neuropsychiatric consequences in five main domains: (1) impairment of attention and (2) emotion and (3) disruption of social skill, (4) psychosis, and (5) autism spectrum disorders [81]. In ASD, there are data that strongly support the structural-functional abnormalities in the cerebellum in patients with autism. Although ASD is adjusted to be resulted from cerebellar dysfunction, it is obvious that multiple regions of the brain undergo dysfunction. Thus, the specific contribution of the cerebellum in the pathophysiology of ASD is needed to be clearly understood. The cerebellum has been demonstrated to modulate and automatize the motor movements, in order to optimize performance [46]. Also, it has been observed that the activation patterns in the primary motor cortex are modulated by transcranial magnetic stimulation of the cerebellum [37]. This shows the cerebro-cerebellar relationship and verifies that alteration in cerebellar activity can affect different regions of the cerebral cortex, influencing internal models of behavior, and optimization and prediction of future behavior [47]. Despite these effects, it does not mean that the cerebellar injury leads to complete loss of its function [79]. To this, cerebellar injury may not include paralysis, but classic motor dysfunction, such as poorly calibrated dysmetric movements, can be occurred. The modulatory effect of the cerebellum is not exclusively related to the motor movement but is associated with impairment of cognition and affect [47]. Moreover, there is region-specific motor dysfunction, as the posterior cerebellar injury demonstrates no severely impaired cognition and language but can lead to disrupted modulation and optimization of cognitive performance such as agrammatism or semantic fluency [79, 80]. These findings emphasized the importance of the cerebellum in implicit learning and skill acquisition, which are directly associated with the process of building and optimizing internal models. The cerebellum is believed to be completely associated with initial motor skill learning, while cortico-striatal pathways and primary motor cortex are more involved in the learned motor behaviors, as well as cognition and working memory [23, 37]. A cerebellar role in learning and skill acquisition is compelling in the neurodevelopment and neurodevelopmental disorders. Indeed, impairment of skill acquisition is more correlated to developmental disorders including ASD, dyslexia, and developmental coordination disorder [10, 96]. These differences are assumed to be related to cerebro-cerebellar

circuits [86]. Thus, behavioral defects resulting from neurodevelopmental disorders are linked to differences in structure-function relationship of specific regions of the cerebellum [112]. For instance, damage of the posterior cerebellar area may result in communication impairments in patients with ASD, whereas motor defects of speech, stuttering, are found to be relevant to overactivation of the anterior lobe of the cerebellum [90]. Deficits of the mentioned cerebellar circuits were observed to cause long-term disorders by influencing the acquisition of motor, communication, and social skills during early neurodevelopment in patients with ASD.

Cerebellum Plays a Role in ADHD

Regarding to the present findings, alteration in structure and function of the cerebellum is believed as the common phenomenon in ADHD [20, 25, 97], but the genetic and environment are thought to be predisposing risk factors of the neurodevelopmental disorder. Genetic investigations have shown that a family-based single-nucleotide polymorphism (SNP) in the XKR4-gene (XK-Kell blood group complex subunit-related family, member 4) in the cerebellum is suggested to be related to incidence of ADHD [57, 69]. Despite the unclear function of this gene in the brain, the importance of this gene was understood by finding that it codes for an inferred protein related to the XK-protein, part of the XK-Kell blood group complex [25, 59, 60]. XK-protein is observed to be widespreadly overexpressed in the brain compared to Kell-protein in the Purkinje cells of the cerebellum [113, 114]. As the linkage between XK gene and McLeod syndrome, a syndrome with sex-dependent defects of central nervous, neuromuscular, and hematologic systems in males including impairment of movement and cognition and psychiatric disorders [19], was found, the hypothesized relationship between XKR4-gene and psychiatric phenotypes was potentiated. It is noteworthy that a correlation exists between XKR4-gene and substance abuse [95], while a SNP in the XKR4-gene has been contributed to responsiveness to antipsychotic therapy [35, 58].

Environmental and epigenetic factors are found to be linked to the cerebellum and its function in prenatal and postnatal stages. Studies of children with ADHD have demonstrated lower pronounced familial effects on the cerebellum volume compared to other regions of the brain [26]. Moreover, in contrast to some reports suggesting that the cerebellum's heritability may be enhanced into adolescence and adulthood [73, 98], the cerebellum is considered as the least heritable brain structures at birth [39] and in childhood [72]. Prenatal adversity may influence the cerebellar development, which begins at early intrauterine life [65, 93, 94]. These show the importance of prenatal and early postnatal period in development of cerebellum to reach a normal structure and function. Unless, negative effects on the cerebellum in patients with ADHD have demonstrated to be relevant to impairment of the cognitive phenotypes, such as temporal processing [27]. However, the role of environmental effects on the cerebellar development and its contribution to the symptoms of the neurodevelopmental disorders are remained to be obviously understood.

Cerebellum Plays a Role in Behavioral Despair and Neuropsychiatric Illnesses

Body of evidences has proposed that there is regionally abnormality in the brain volume in patients with major depressive disorder (MDD). Several meta-analyses have confirmed this hypothesis that a reduction in gray matter volume (GMV) of dorsolateral prefrontal cortex (DLPFC), anterior cingulate cortex (ACC), and hippocampus was observed in patients with MDD [12, 22, 24, 55, 108, 110]. The reports have suggested two disorders as the pathophysiological factors of MDD, as below:

- Impairment of structure and function within cortico-limbic circuitry [49]
- Alterations in the functional organization of multiple brain networks implicated in attention regulation, emotional processes, and cognitive control [49]

Although the involvement of the cerebellum in both cognitive and affective processes is now well established, meta-analyses show no significant and obvious contribution of the cerebellum in MDD. The studies indicated that the linkage of the cerebellum with cerebral cortices and paralimbic regions indeed, as cortico-cerebellar circuits, is key point to clarify the role of the cerebellum in MDD [81, 88]. The limited data on the involvement of the cerebellum in MDD may be related to the few investigations of cerebellar structure in MDD. However, the analytical studies were focused on the vermian volume and lack of gray/white matter parcellation [107]. Moreover, clinical evidences reported an abnormal structure of the cerebellum in depressed patients using whole-brain investigations of altered GMV in depression [22, 56, 71, 99, 107].

To better understand the role of the cerebellum in behavior, fMRI data were analyzed in adolescents and young adults to identify the possible association between emotional and behavioral disorders with brain areas [76]. Interestingly, the results emphasized that the cerebellum, as well as cerebral sensorimotor and limbic areas, had the strongest link to behavioral despair.

In addition to MDD, the investigations demonstrated a significant association between obsessive-compulsive disorder (OCD) and abnormalities in the cerebellum. There were found significant, obvious abnormalities in the cerebellum, along with in the temporo-parieto-occipital and fronto-striatal areas in patients with OCD compared to healthy controls [44]. Although we have limited data on the role of the cerebellum in pathogenesis of anxiety disorders, accumulation of evidence of the importance and involvement of the cerebellum in a wide variety of psychiatric and neurodevelopmental disorders are needed to be elucidated [3].

Schizophrenia, as known as neurodevelopmental disorder with uncertain etiology, is thought to be associated with the cerebellum, which has been considered as a proposed target of the neurodevelopmental processes. The schizophrenic phenotype consists of a variety of neuronal and behavioral disorders. Also, it includes the impaired cognition, termed as “cognitive dysmetria” that involves the thought form. The literature proposed that this condition may be relevant to the pathological status of the cerebellum [105]. The brain regional analogy has also demonstrated that

deficits in the cerebellar cognitive or affective circuits may lead to thought disorder and/or tangentiality. The investigations using longitudinal and cross-sectional structural MRI proposed the implication of cerebellar development in schizophrenic patients with childhood onset and compared the resulted data to healthy controls [3, 50]. The results showed a decrease of the volume of the cerebellum and cerebrum in adolescent patients with schizophrenia. Moreover, Greenstein et al. [41] explored abnormal different trajectories of cerebellar development in patients with childhood-onset schizophrenia.

Conclusion

Body of evidences has found a critical role of the cerebellum in the development of motor and non-motor (e.g., cognition and behavior) conditions, which was disrupted by cerebellar injury in preterm infants, developmental cerebellar lesions in infants, cerebellar tumor in pediatric patients, and neurodevelopmental defects. As developmental differences occurred in cerebellar malformations and neurodevelopmental disorders, it is thought to be associated with motor, cognitive, and behavioral dysfunction. The cerebellar injury in preterm infants could enhance the rate of cognitive and socio-behavioral dysfunction. Consistent with preterm newborns, cerebellar tumor resulted in similar motor, cognitive, and behavioral defects in pediatric patients. Furthermore, the region-specific lesions may determine the effects of early cerebellar damages on the neurodevelopmental and behavioral disorders. The cerebellar dysfunction at early life can cause distinct, long-term effects on the brain distal areas which are projected by the cerebellum. The developmental diaschisis can influence the structure-function relationship of the regions of cerebral cortex which may be optimized by the cerebellar input. In summary, increasing clinical and neuroimaging evidences in newborns that undergone acquired and developmental cerebellar lesions, along with older children with cerebellar damage, presented novel approach to the role of the cerebellar lesions at early life on cerebral development. On the other hand, determination of the age of cerebellar injury to the developing brain may help us to predict the possible long-term outcomes (Fig. 1).

However, the effects of cerebellar lesions at prenatal and postnatal periods on cerebral development should be clarified. Further studies are needed to better understand the structure-function relationship in the developing cerebellum to improve clinical prognosis, early intervention services, and educational planning. The findings can open a new avenue to explore novel treatment of the cerebella injury-induced neurodevelopmental and behavioral disorders by cerebellar neuromodulation. It is also possible that therapeutic interventions, such as cerebellar neuromodulation, could provide alternate treatment options in these populations. Growing of our knowledge of the association between cerebellar circuits and specific behaviors can facilitate to reach to point of optimization of timing and localization of the therapeutic strategies. These essential findings will guide us to improve the life of millions of children impacted by cerebellar injury and the subsequent developmental disorders.

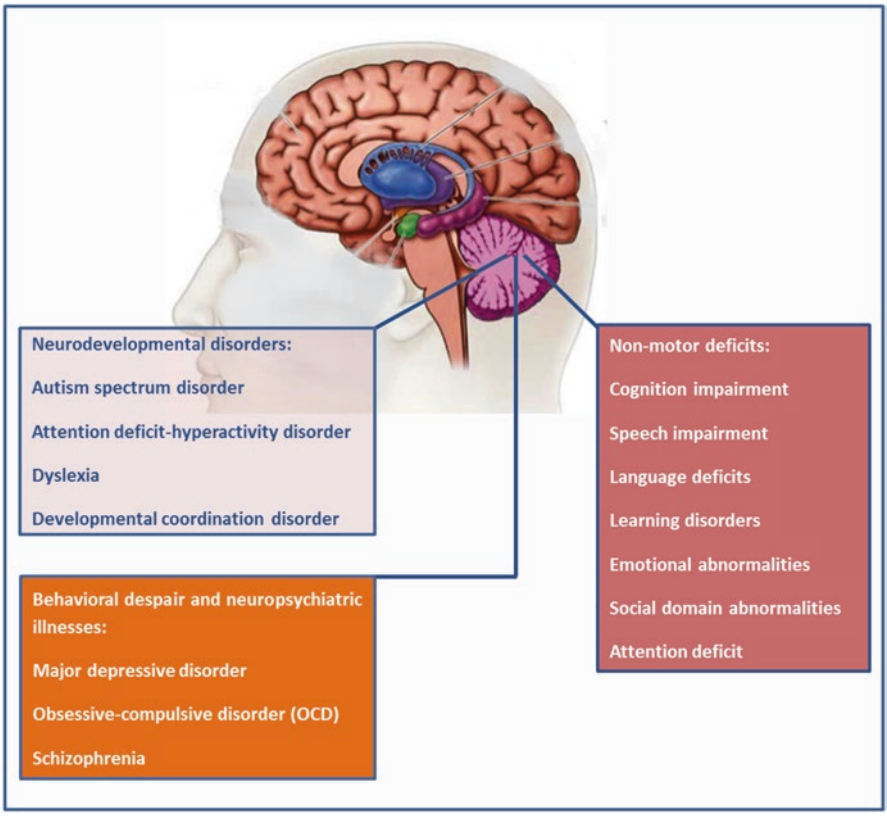

Fig. 1 Schematic of the cerebellum and its associated non-motor, neurobehavioral, and behavioral disorders. The cerebellar damages and dysfunction may lead to a variety of non-motor deficits and behavioral outcomes in patients with neurodevelopmental disorders

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Neurodevelopmental Disorders of the Cerebellum: Autism Spectrum Disorder

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Abstract Autism spectrum disorder (ASD) is a neurodevelopmental disorder with an incidence of 1 in 68 children. Cerebellar abnormalities have been observed in many ASD patients. The cerebellum is an elaborate brain region critically important for motor learning and coordination of movement, and increasing lines of evidence indicate that the cerebellum also contributes to emotion and cognition. In this chapter, we will review the genetic and environmental factors that may cause cerebellar deficits in ASD patients. Structural and functional cerebellar abnormalities based on neuroimaging and histopathological studies and current approaches to management will be discussed.

Keywords Cerebellum • Neurodevelopmental disorders • Motor skills • Language • Cognition • Autism spectrum disorder

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Introduction

Autism is a commonly occurring complex neurodevelopmental disorder. Leo Kanner, a child psychiatrist (1943), first described patients with "a powerful desire for aloneness" and "an obsessive insistence on persistent sameness" as "early infantile autism" [1–4]. A similar behavioral disorder, "Asperger's syndrome," was reported by Hans Asperger [5]. To avoid using different terminologies, these disorders were together named "autism disorders" in 1987. Recently, the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) collectively designated all autism-like disorders as "autism spectrum disorder" (ASD) [3].

ASD is characterized by a triad of symptoms: (1) impairment in social interaction, (2) communication difficulties, and (3) restricted, repetitive, and stereotyped patterns of behavior [3, 6–10]. Current methods can diagnose the ASD in children as young as 2 years old, and males are four times more likely to be diagnosed with ASD than females [11]. The prevalence of ASD is estimated at 1 in 68 children in the United States [12] and 1–2% in Asia, Europe, and North America (see chapter "Epidemiology of Cerebellar Disorders"). The etiology of ASD is complicated: in some patients it is unknown, and in some cases, individuals are affected due to gene mutations and/or environmental factors [7]. However, the interplay of genetic, environmental, and epigenetic factors probably underlies the mechanisms of ASD [8, 13].

A subset of ASD patients, about one in five, display increased head circumference and brain volume in early childhood, typically until 5–6 years of age [14, 15]. In these patients, the cortical white matter, the thickness of the corpus callosum, and the volume of cerebrospinal fluid (CSF) in the subarachnoid space are increased at the age of 6–9 months [16–18]. The frontal cortex has been reported to be larger, probably due to increased neuronal density in the prefrontal cortex [19]. Other brain regions that are prominently implicated in ASD include the cerebellum; brainstem and limbic system, including the hippocampus; and basal ganglia [20]. These areas are most likely responsible for the symptoms of those patients with abnormalities related to social behavior, executive functions, atypical use of language, and difficulties with speech [21]. Additionally, enlargement of the amygdala and caudate nucleus may cause anxiety and repetitive behavior [22].

Recent advances in molecular genetics and imaging technologies have shown that the cerebellum is one of the most consistently affected brain regions in ASD patients [8, 23, 24]. The cerebellar neurodevelopmental deficits in ASD include abnormalities in the cerebellar cortex, neurodegeneration, and impaired cerebellar circuits. Together, these deficits affect motor, sensory, language, and cognitive functions [25–28].

Autism Spectrum Disorder Pathogenesis

Emerging evidence from genetic association studies and postmortem human brain tissue indicates that ASD is either hereditary or caused probably by de novo mutations in a number of genes. Additionally, certain environmental risk factors have

been proposed to be causative in ASD. The set of molecular pathways, neural circuits, and behaviors affected in the different autisms is highly complex, and as a result, it has been difficult to uncover the neurobiological underpinnings of ASD [9, 29]. How the cerebellum contributes to the etiology of ASD has been particularly underappreciated.

The cerebellum develops from early embryogenesis to the first year postnatally in human. This long period of pre- and postnatal cerebellar development makes it susceptible to many risk factors [30–34]. In this section, we briefly review the findings regarding currently identified genetic and environmental risk factors in ASD. Epigenetic susceptibility factors have been discussed in chapter "Epigenetics and Cerebellar Neurodevelopmental Disorders."

Genetic Factors

Several lines of evidence have revealed that ASD is a neurodevelopmental disorder determined largely by genetic factors [35]. For example, twin studies have higher concordance rates for monozygotic twins than for dizygotic; approximately 80% of monozygotic twins are concordant compared to 10% of dizygotic twins, with a heritability of over 90% [36]. Recently, genome-wide association studies have identified many genes as risk factors for ASD. These genes span several chromosomal loci, and many are highly expressed in, and are involved in, the development of the cerebellum [37, 38]. Sadakata et al. [38] categorized these genes based on their role in development of the nervous system and synapse development and function. Some of these genes, such as CDH9, CDH10, RELN, and PTEN, are involved in developmental process such as neuronal differentiation, migration, and circuit formation. An important category of ASD-associated genes regulates synaptic adhesion and synaptic transmission, including genes encoding for neurexins, neuroligins, leucine-rich repeat transmembrane neuronal proteins (LRRTMs), Shanks, and SynGAP [39–42]. Another category of ASD risk genes encodes for proteins required for transcription and translation such as EN2, TSC1, FMR1, and MECP2 [38, 43].

Chromodomain-helicase-DNA-binding protein 8 (CHD8), previously called Duplin, is one of the genes most strongly associated with ASD [44, 45]. It was the first identified in the screen for novel interactors within the canonical Wnt/β-catenin pathway [46]. CHD8 is an ATP-dependent chromatin-remodeling factor [47] and may serve as a "master regulator" for other ASD risk genes during fetal development [44, 48]. Knockdown of CHD8 in human neural stem cells affects the expression of several ASD risk genes [44], and human patients with mutations in CHD8 display ASD symptoms and have macrocephaly and gastrointestinal difficulties [37]. Taken together, these data suggested that CHD8 targets a set of genes during brain development and regulates other ASD risk genes [44]. Some of the ASD risk genes regulate developmental processes in the cerebellum [13]. These include genes encoding for Reelin, RORα, EN2, BDNF, neuroligins, and neurexins [8, 49].

RELN dysregulation has been observed in a subset of autistic individuals (reviewed by Ishii et al. [36, 49]). Reelin, encoded by the RELN gene (located on chromosome 7 in human and chromosome 5 in mice), is a 388 kDa extracellular matrix glycoprotein which is essential for proper neuronal migration and positioning during embryonic and perinatal development of the brain/cerebellum [8, 36]. Though the precise mechanisms of RELN's role in ASD pathogenesis is uncertain, trinucleotide repeat expansion in the RELN gene has been observed in autistic individuals [8, 49]. Persico et al. (2001) first reported that the polymorphic GGC repeats located in the 50 untranslated region (50 UTR) of the RELN are associated with ASD disorder [50]. The finding was subsequently replicated in three studies [51–53], but there were no confirmed association between the triplet repeats in the 50 UTR of the RELN and autism. The family-based association analyses revealed that many CGG repeats present in RELN alleles may cause ASD particularly in patients with speech difficulties [54].

Reelin mutations in mouse models lead to irregular cortex formation and abnormal layering which may be responsible for behavioral and neurological disorders [55]. Adulthood changes in Reelin protein level caused cognitive impairment and reduced synaptic plasticity [55–58]. Given that the genetic evidence implicates RELN in the etiopathology of ASD, it has been attempted to add biochemical evidence by measuring the Reelin level in brain tissue and blood by using Western blotting. They showed that the levels of Reelin were significantly reduced in patients with ASD [59].

Several lines of evidence indicate that genes encoding retinoic acid receptor-related orphan receptors (RORs) are also associated with ASD. The RORα, RORβ, and RORγ are nuclear receptors that regulate a range of physiological processes during brain development [60–62]. RORα and RORγ are broadly expressed in the body, whereas RORβ expression is more restricted to the central nervous system [62, 63]. RORα protein expression significantly decreases in the brains of ASD patients probably through epigenetic alterations [64]. Devanna and Vernes demonstrated that miR-137, a microRNA implicated in neuropsychiatric disorders, targets a number of genes associated with ASD including RORα [65]. RORα is a transcription factor that is critically important for development of the cerebellum [60, 61, 66]. The role of the RORα in neural development has been demonstrated in mouse strain *staggerer*, which harbors a spontaneous deletion within RORα [67]. These mice have small stature and develop ataxia and hypotonia. The major neural deficit was underdevelopment of the cerebellar cortex with a pronounced deficiency in both granule and Purkinje cells [67]. Furthermore, disruption of RORα in *staggerer* mice shows behavioral phenotypes such as abnormal spatial learning, reduced exploration, limited maze patrolling, and perseverative behavior, which are associated with ASD [61, 62].

Engrailed 2 (EN2), a homeobox transcription factor, has been associated with normal cerebellar development, and mutations or deletions of EN2 result in reduced cerebellum volume and structural abnormalities [68, 69], which are both associated with susceptibility to ASD [70]. Brain-derived neurotrophic factor (BDNF) plays a key role in the development of the nervous system and modulation of neuronal activity, both of which impact complex human behaviors. Several studies have been performed to measure peripheral blood levels of BDNF in an attempt to

find a biomarker for children with ASD. Peripheral blood levels of BDNF are known to be highly correlated with brain BDNF levels [71]. Although there is no consistency in the association between BDNF levels in blood and ASD, a recent review by Xiao-Yan et al. (2016) using meta-analysis indicated that there are increased peripheral blood levels of BDNF in ASD patients [72]. Furthermore, $Ca2^{+}$-dependent activator protein for secretion 2 (CADPS2) contributes to normal cerebellar development by enhancing release of BDNF and neurotrophin-3 (NT-3) [73, 74]. The CADPS family is a secretory-related protein family that regulates secretory granule exocytosis, which in vertebrates consists of two genes, CAPS1/CADPS1 and CAPS2/CADPS2. The expression level of the CAPS2 has been observed to be unusually high in some patients with ASD [38, 75].

Mutations in the methyl CpG-binding protein 2 (MECP2) gene are known to cause Rett syndrome, a disorder characterized by language impairments, motor deficiencies, and stereotypical behavior [76], which is under the umbrella of ASD. Patients with Rett syndrome frequently have cerebellar atrophy that increases with age [13] (see chapter "Epigenetics and Cerebellar Neurodevelopmental Disorders").

Tuberous sclerosis complex (TSC) is a genetic disease that causes benign tumors in the body, including the brain [77]. Mutation in the TSC1 and TSC2 genes causes TSC with a neurodevelopmental disorder that involves higher rates of ASD [77, 78]. TSC produces a protein that negatively regulates the target of the rapamycin (mTOR) signaling pathway to control molecular and cellular process. Tsai et al. (2012) designed a mutant mouse model in which the gene for Tsc1 is not expressed in Purkinje cells [78]. These mutant mice displayed ASD-like behaviors, such as abnormal social interaction and ultrasonic vocalization, and inflexibility. In addition, recent discovery has shown that the granule cells/Purkinje cells are important for cognitive processing in the cerebellum [79]. These studies are significant because they demonstrated a clear involvement of the cerebellum in non-motor functions as well [78].

Environmental Factors

It has been suggested that the risk of developing ASD increases with exposure to environmental factors such as teratogenic substances (e.g., thalidomide, valproate, and misoprostol), infection with viruses (e.g., influenza, rubella, and cytomegalovirus) during pregnancy, and advanced age of parents (for reviews, see references [38, 80, 81]).

Some environmental risk factors such as exposure to valproic acid during prenatal development may cause abnormalities in cerebellar development and ASD [82]. In rat, valproic acid exposure reduces the number of Purkinje cells in the cerebellum accompanied by increases in the number of apoptotic cells [83]. Cole et al. (2011) have shown changes in cerebellar gene expression in mice treated with chlorpyrifos [84]. Dermal exposure of young adult mice to chlorpyrifos causes increased glial fibrillary acidic protein expression of the cerebellum [85]. Furthermore, Purkinje cell numbers are reduced in rats prenatally exposed to chlorpyrifos [86]. Other factors

such as organophosphate pesticides and antiepileptic drugs have been shown to affect cerebellar development and potentially cause ASD [87].

Maternal fever is another environmental risk factor that affects the cerebellum and leads to apoptosis. It also interferes with neuronal maturation and may cause heat shock protein activation during cerebellum development in ASD [88–90].

Viral infections can affect cerebellar and neocortical development during pre- and neonatal and cause neuropathy in ASD [91, 92]. Influenza virus also has the same impact on cerebellum development such as reduced the number of Purkinje cells and interruption in migration of Purkinje and granule cells during perinatal development, which may cause deficits in working memory and behavioral impairments [93–96] (see chapter "Infections of the Cerebellum").

Functional gastrointestinal disorders (FGIDs) are disorders independent of organic or physiological conditions that are the most common causes of GI disorders in children with ASD. FGID symptoms include abdominal pain, constipation, irritable bowel syndrome, and functional dyspepsia [97]. The FGIDs are associated with impaired behaviors and sensory responses, as well as changes in sleep patterns [98]. It is suggested that inadequate brain-gut interactions may be responsible for these symptoms in ASD patients [97]. Changing the gut microbiome to treat the ASD behaviors such as anxiety and depression is a new line of study that hopes to find alternate treatments for ASD patients [99, 100].

The exposure to air pollution, which may cause immune responses, is another likely environmental risk factor for ASD [101]. The immune response results in activation of immune cells and antibody production and increases the leukocyte migration to the brain tissue by increasing diffusion through the blood-brain barrier. It is suggested that maternal immune activation at a critical time points impair cerebellar morphology and a variety of motor and non-motor behaviors [102]. The abnormal level of blood immunological markers in ASD patients is evidence of interactions between genetic/environmental factors with their immune system in these patients is shown [103, 104] (see chapter "Neuroimmune Mechanisms of Cerebellar Development and Its Developmental Disorders: Bidirectional Link Between the Immune System and Nervous System").

Diagnosis of ASD

Studies on patients with ASD using advance brain imaging, genetic, and behavioral observations improved our knowledge of ASD symptoms. As of yet there are no biomarkers for the diagnosis of ASD, and currently clinical diagnosis of these patients is based on behavioral observations combined with patient history [22, 105]. Three ASD diagnosis criteria – social reciprocity, communication, and restricted/repetitive behavior – have been published by *DSM*-IV. However, it recently has been revised by *DSM*-V and International Classification of Diseases, Tenth Edition (ICD-10), into two domains of diagnosis criteria, (1) deficits in social communication/interaction and (2) restricted and repetitive behaviors, with

evidence of persistent symptoms that cause functional impairment [105]. Murphy et al. (2016) highlighted three key issues, sleep, GI problems, and epilepsy, regarding physical health that may be important in the diagnosis of ASD patient [105]. The mental health issues are present both in adults and children with ASD, which include mood and anxiety disorders, obsessive-compulsive disorder (OCD), attention-deficit hyperactivity disorder (ADHD), and psychotic disorders. These persist from childhood to adulthood in both sexes. Additionally, ASD patients have specific cognitive anomalies, including poor planning, decision-making, timing, and motor skills, which impact their daily activities [105–107].

The Cerebellum and ASD

Cerebellar malformations have been associated with a range of developmental impairments and behavior disorders including ASD (see review by Bolduc and Limperopoulos [31]). Motor impairment and clumsiness has been noted as an important feature in ASD [108]. It is shown that about 80% of children with ASD have motor coordination deficits, which have a positive correlation with the severity of the ASD and intellectual disabilities [109, 110]. Cerebellar motor dysfunction in ASD includes eye movement abnormalities, fine and gross motor deficits, gait, balance and coordination impairment, postural instability, and motor learning deficits [109, 111]. Motor impairments are among the earliest signs of an autistic phenotype [112]. It has been shown that motor impairments are predictive of the ASD outcome. During early movement activities, individuals, who are later diagnosed with ASD, have poor fine and gross motor skills that are accompanied by delays of language development [109, 113]. Similarly, difficulties in oral and manual motor skills in infancy can label individuals as an ASD patient, and late speech fluency is predictable [114]. In addition, early motor delays are more common in infants at risk for ASD and are related to later communication delays [115]. Therefore, the timing of language acquisition may serve as an indicator for neurodevelopmental and behavior disorders and may be a marker to diagnose people with ASD.

Emotional/behavioral disturbance and communication disorders may be associated with the motor task performance in ASD patients [116]. It has been suggested that the lack of gesture and imitation in ASD patients might be related to motor dysfunction, providing a mechanism by which cerebellar dysfunction could impact the core social communication symptoms of patient with ASD [109, 117].

ASD and Cerebellar Structure Abnormalities

Cerebellar abnormalities are the most consistently reported brain structural changes in ASD, such as decreased cerebellar cortex, which is a key landmark for diagnosis in ASD brains [109, 118]. Cerebellar enlargement has been reported in ASD young

children in comparison with the total brain volume and may be associated with the cerebellar white matter [26, 119, 120]. However, the growth rate declines later during development and eventually results in a smaller cerebellar volume by adulthood in ASD patients [120, 121].

By MRI, cerebellar lobules VI and VII are hyperplastic in patients with ASD, and it is suggested that these alterations may be responsible for increased stereotype and repetitive movements [122]. The language impairment in ASD may be associated with a decreased volume of the vermis and anterior lobe and abnormal left lateralization in lobule VIIIA [123, 124]. A study conducted using voxel-based morphometry suggested that structural differences such as increase and decrease in cerebellar gray and white matters are related to specific abnormalities at the different stages of cerebellar development in ASD patients [109, 125].

Neurohistological studies show changes in the anatomy of the cerebellum in patients with ASD, including a decrease in the number of Purkinje cells [126, 127], immature cerebellar development [128–130], morphological changes in the size of the cerebellar nuclei which are small and abnormal, and an increase in the number of Bergmann glia cells [131, 132]. The low density of the Purkinje cells in the cerebellum of ASD patients was observed in the vermis, Crus I–II, lobules IV–VI, and lobule X [133]. The smaller size of Purkinje cells may indicate the occurrence of an atrophic process [134]. Because of the large size, and due to numerous synapses with the parallel and climbing fibers, Purkinje cells have a high metabolic demand. Therefore they have extensive amounts of calcium storage that may cause increases in intracellular calcium, which elevates the risk of excitotoxicity and cell death [135].

It has been reported that the cortical-pontine-cerebellar-thalamic circuit is immature and abnormal both functionally and anatomically in patients with ASD [9]. It is also shown that the cerebellar input and output pathways in relation to neocortical areas are unusual in ASD patients [136, 137]. The corticopontocerebellar pathway carries inputs that originate from the primary sensory and motor cortex, posterior parietal, prefrontal, orbitofrontal, cingulate, temporal, and basal nuclei and projects to the cerebellum [138, 139]. Outputs originate from the cerebellar nuclei and through the thalamus project to the neocortex [140–142]. These circuits are specialized for cognitive and behavioral activities such as executive functions, language, and emotions. Thus, the cerebellum may be responsible for cognitive impairment, sensorimotor behavior, and social disconnection in ASD [13].

Eye gaze abnormalities during social interaction are an early diagnostic indicator in ASD patients. Gaze fixation is naturally used to fix the fovea on an image or object. The oculomotor system maintains fixation, which is supported by the nuclei of the brainstem. Therefore, inputs from the frontal eye fields and superior colliculus actively block the saccades away from the object of interest [143]. The pontine nuclei stimulate Purkinje cells in lobules VI–VII vermis cerebellum, and inhibitory outputs from the oculomotor vermis help to stop undesired eye movements and keep an image on the fovea [144] which could potentially be used as an early marker of ASD patients.

Control of upper limb movement is related to the frontoparietal cortex as well as the cerebellar cortex and its output nuclei [145]. Upper limb and manual motor deficits that are associated with atrophy of the intermediate and lateral cerebellum

involve lobules I–V as well as more lateral areas of lobules V–VI extending into Crus I–II in patients with upper limb ataxia. [146]. These loops are responsible for regulating the amplitude, duration, and timing of movements [147, 148]. Patients with ASD have difficulties coordinating grasping and reaching activities [149]. These difficulties may be caused by central defects in integrating sensory feedback information and motor output as well as deficits in neocortical-posterior cerebellar circuitry. The compromised motor learning in individuals with ASD could be related to disturbances in the anterior cerebellar lobules IV–VI and their connectivity to frontal as well as parietal regions of the cortex. These effects may damage upper limb and manual motor actions that ultimately impact the patient's ability to control motor behavior and learn new skills. Therefore, the development of more complex social motor skills in these patients is disabled. Medial and intermediate cerebellar circuits affected by insufficiency in both sensory feedback and forward control appear to cause motor impairments and difficulties in posture, gait, and walking in ASD patients [150]. The motor deficits start from infancy and extend to adolescence and adulthood [108, 151–153].

Cognitive function deficits such as attention and memory impairment, executive function, and cognitive flexibility deficits are common features in ASD [154]. The cerebellum communicates with Brodmann areas 46 and 9 of the prefrontal cortex, which are involved in cognitive functions, memory, planning, decision-making, and cognitive flexibility [155–157]. The cerebellum to prefrontal cortex pathway could affect cognitive functions directly or perhaps indirectly through the ventral tegmental area, which contains dopaminergic neurons that project and terminate in the prefrontal cortex [158]. Notably, the function of the prefrontal cortex dopaminergic pathway is associated with attention selection, cognitive flexibility, and memory [157]. A maldevelopment of connectivity in connection of the cerebellum to this higher-order circuit may explain the cognitive involvement of the cerebellum in patients with ASD.

Assessment and Treatment

There is very limited accurate and practical information to assess, diagnose, and manage ASD conditions. Therefore, because the number of ASD patients has rapidly increased during past decade, there is an urgent need to improve knowledge and develop assessment tools and treatment of ASD patients [105].

ASD diagnosis can be difficult because of a large amount of heterogeneity, varying presentation, and variability in symptoms [159]. There are no biomarkers to diagnose ASD; therefore behavioral presentation of the patients is used for diagnosis [160]. The gold standard for clinical diagnosis in these patients is based on current diagnostic classification systems and proceeding very careful assessment practices. These assessments include physical examination, hearing test, observation of children's behavior, and a structured parent interview that covers the patient's full developmental history [160]. Currently, the best practice to diagnose ASD patients is a step-by-step strategy that is recommended by the American

Psychological Association [161]. This diagnostic strategy starts with child's parent/caregiver concern and followed by a formal diagnostic assessment conducted by a pediatrician or/and appropriate referrals. The formal diagnostic assessment includes medical and functional evaluation such as everyday verbal and nonverbal skills and level of ability as well as analyze/assess of behaviors based on developmental aspect [162]. However, because of differences in cognitive function, age, language level, and the source of information, diagnosis of ASD is very difficult [159].

Children who are diagnosed with ASD need to be reevaluated continuously during preschool years to identify their weakness, inabilities, and difficulties [159]. There are also some diagnostic instruments for ASD such as the Autism Diagnostic Observational Schedule – Generic (ADOS-G) [163], which assesses communication, play, and creative use of materials and possibilities for children who may have ASD. The best Screening Tool for ASD in Toddlers and Young Children (STAT) [164] is structured to identify children between 24 and 36 months of age with ASD. One of the measures of early communication in children 8–24 months is the Communication and Symbolic Behavior Scales (CSBS) [165]. Additionally for the parent interview, there is a clinical diagnostic instrument named the Autism Diagnostic Interview-Revised (ADI-R) that is addressing early development, communication/language, social interactions/interests, and restricted and repetitive behaviors [166]. The Social Communication Questionnaire (SCQ) is an appropriate method to get information from parents [167].

Usually an assessment starts with medical evaluation which will be conducted by physicians and, if the ASD suspected, referring the patient for diagnostic assessment as well as considering the pediatrician as an alternative referral. When diagnosis is confirmed, treatment planning should involve the professional health team [159].

Summary

Many genetic and environmental factors may cause ASD. The mechanisms are unknown, but presumably genetic and environmental factors affect normal brain development and, consequently, lead to functional disorders in patients with ASD.

There is mounting evidence that developmental abnormalities in the cerebellum may underlie the pathogenetic mechanisms that are associated with the ASD phenotype. Cerebellar developmental disorders that are associated with ASD pathogenesis show deficits in motor coordination, balance, motor memory, and higher-order dysfunctions including speech and attention regulation.

The major goal of management in ASD patients is early diagnosis for behavioral and medical interventions to enhance the functional ability of these children. The new approach involving brain-gut-microbiome interactions may provide a biomarker associated with GI disorders that could be helpful in the early diagnosis of these patients. Because the number of ASD patients is increasing, studies are needed to develop assessment tools and treatment, increase public awareness, and develop strategy for health care of patients with ASD.

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Clinical Aspects of the Inherited Cerebellar Malformations

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Abstract Inherited cerebellar malformations cause lifelong disability and are not well studied in the newborns because there is a lack of appropriate clinical examination tools. Recently, inherited cerebellar malformations have been investigated using emerging advanced neuroimaging technology such as MRI, which revealed many cerebellar developmental disorders. These malformations cause impairments that involve motor and non-motor functions. Cerebellar hypoplasia, Dandy–Walker syndrome, Joubert syndrome, pontocerebellar hypoplasia, and rhombencephalosynapsis are examples of cerebellar malformations. In this chapter we will focus on cerebellar malformations that have been reported using characteristic symptoms and signs. The current approach for evaluation of the affected patients, differential diagnosis, and management will be discussed.

Keywords Cerebellar imaging • Cerebellar disorder • Cerebellar hypoplasia • Dandy–Walker syndrome • Joubert syndrome • Pontocerebellar hypoplasia • Rhombencephalosynapsis

Introduction

The cerebellum cytostructure is discussed in chapter "The Embryology and Anatomy of the Cerebellum." Cerebellar development begins during an early embryonic stage with a complicated developmental process that continues well into the first year after birth in humans. Recent advances in neonatal intensive care,

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neuroimaging techniques such as positron emission tomography (PET), magnetic resonance imaging (MRI), and functional MRI (fMRI) have improved our ability to understand the structural and functional anomalies that implicate cerebellar involvement in numerous motor and non-motor functions, ranging from motor/sensory integration and working memory to various higher-order cognitive processes [1–4]. Despite the advanced technology, understanding cerebellar malformations in children requires additional research regarding their prognosis, and they have lifelong consequences. Because of a lack of appropriate treatment, up to 80% of parents choose to terminate pregnancy after a prenatal diagnosis of a cerebellar malformation [1, 5]. The prolonged developmental process in the cerebellum makes it more vulnerable to perturbation caused by genetic factors, environmental insults, or a combination of both that occurs during development. Cerebellar abnormalities range from subtle impairments including cognition to significant structural defects with life-threatening or lifelong disabilities. Before the introduction of MRI, Dandy–Walker variants were a term used to characterize several types of cerebellar malformations.

Cerebellar dysfunction disturbs the regulation of muscle tone, motor control, and coordination of movement, which is called ataxia—a broad term that refers to a disturbance in the smooth performance of the motor activities. The non-motor dysfunction that results from cerebellar manifestations includes cognitive affective syndrome that includes impairment in executive function, spatial cognition, personality changes, and language deficits [6–9]. Cerebellar structural and functional abnormalities have been reported in psychiatric disorders such as schizophrenia, bipolar disorder, depression, anxiety disorders, attention deficit hyperactivity disorder (ADHD), and autism [10–15].

The specific constellation of symptoms is sometimes useful for localizing the cerebellar lesion, but often there is considerable overlap. Because of a complex developmental process during cerebellum formation, clinical classification of cerebellar neurodevelopmental disorder is difficult; however, a classification has been suggested that is based on embryological and genetic considerations [16].

Cerebellar malformations can either be primary or secondary. In the latter group, the cerebellar defects are secondary to a developmental disorder in structures around the cerebellum such as Chiari malformation or vein of Galen malformation. Chiari malformations (Fig. 1) are posterior cranial fossa defects that range from herniation of the cerebellar tonsils through the foramen magnum to complete agenesis of the cerebellum. Chiari malformations are classified into four types (I–IV), with type IV being the most severe malformations [17]. The interruption of the surrounding mesodermal development causes congenital hypoplasia of the posterior cranial fossa, and therefore part of the cerebellum herniates through the foramen magnum. Other conditions sometimes associated with Chiari-type I malformation include hydrocephalus, bone abnormalities such as craniosynostosis (especially lambdoid craniosynostosis), hyperostosis (craniometaphyseal dysplasia), and X-linked vitamin D-resistant rickets, syringomyelia, spinal curvature, tethered spinal cord, and connective tissue disorders such as Ehlers–Danlos syndrome and Marfan syndrome [18–21]. Because of familial clustering in some cases of Chiari-

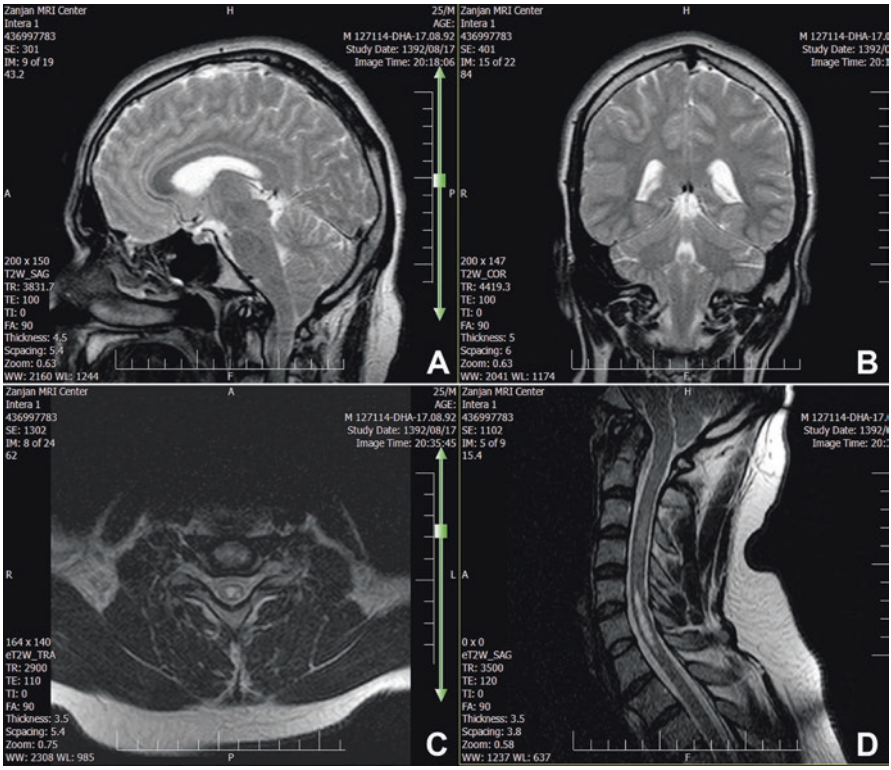

Fig. 1 Chiari malformation type I. (**a**, **b**) Sagittal and coronal T2-weighted brain images. There is slight inferior herniation of cerebellar tonsils through the foramen magnum that is less than 5 mm, and it shows benign tonsillar ectopia that could be a mild variant of Chiari malformation. (**c**, **d**) Axial and sagittal T2-weighted MRI of the brainstem and cervical spinal cord. Note the presence of a large syrinx in association with tonsillar ectopia in Chiari malformation type I

type I malformation, a genetic susceptibility such as gene mutations in chromosomes 9 and 15 has been suggested [22]. In Chiari malformation type II, both cerebellar vermis and tonsil herniation accompany a lumbar or lumbosacral myelomeningocele [23, 24]. The hypoplasia in Chiari-type IV malformation corresponds to primary cerebellar agenesis [25, 26].

Another example is the vein of Galen malformation that results from the presence of one or more arteriovenous fistulas, which constitute up to 30% of intracranial vascular malformations presenting among pediatric patients [27, 28]. The left-to-right cardiac shunt causes a noncyanotic flow condition with resultant heart failure and accompanying macrocephaly. In patients with vein of Galen malformation, the superior cerebellar arteries also discharge into the vein of Galen [29]. It is reasonable to assume that the dilated vein causes direct compression of cerebrospinal fluid (CSF) flow, increased intracranial pressure, and caudal displacement of the cerebellar tonsils [30], which leads to cerebellar signs and symptoms.

In this chapter, we summarize primary cerebellar malformations and discuss current treatment in these patients. Based on our clinical experience and available knowledge, there is no appropriate treatment and most of the patients will be managed conservatively (see chapter "Clinical Features, Assessment, and Management of Patients with Developmental and Other Cerebellar Disorders"). Treatment depends on the specific symptoms and requires a team of specialists including neonatologists, pediatricians, neurologists, and therapists. It is important to refer families of affected children for genetic counseling. In this chapter, we include selected primary cerebellar malformations such as the cerebellar hypoplasia, Dandy–Walker malformation (DWM), Joubert syndrome (JS), and the related diseases pontocerebellar hypoplasia (PCH), rhombencephalosynapsis (RES), lissencephaly, cerebellar dysplasia, and dysplastic cerebellar gangliocytoma or Lhermitte–Duclos disease (LDD).

Cerebellar Hypoplasia

The cerebellar primordium emerges at approximately 28 days postfertilization in humans (embryonic day 7–8 in the mouse) as a neuroepithelial swelling of the rostral lip of the fourth ventricle, which is part of the alar plate of the metencephalon (rhombomere-1) [31–33]. Therefore, any developmental dysregulation that targets the rhombomere-1 causes failure to specify the anterior hindbrain and results in cerebellar aplasia/hypoplasia because of defects in dorsal patterning mechanisms [34–36]. Cerebellar hypoplasia is a heterogeneous group of disorders that was first reported by Crouzon in 1929.

The causes of the cerebellar hypoplasia are broad and include chromosomal aberrations (such as trisomy 9, 13, and 18), metabolic disorders [37], teratogens (drugs and infections; see chapter "Teratogenic Influences on Cerebellar Development"), or isolated genetic cerebellar hypoplasia (such as reelin receptor, very low density lipoprotein (VLDL) [38], dyskerin pseudouridine synthase 1 (DKC1) [39], oligophrenin 1 (OPHN1) [40], pancreas-specific transcription factor 1a (PTF1A) [41], and carbohydrate-deficient glycoprotein syndrome types I and II (CDG1 and 2) [42, 43]). Similar to most developmental anomalies, cerebellar hypoplasia may be associated with other brain malformations and there may be multi-organ involvement. Patel (2002) suggested a classification in which cerebellar hypoplasia is divided into focal hypoplasia (e.g., isolated vermis or hemisphere hypoplasia) and generalized hypoplasia with and without an enlarged fourth ventricle [44, 45].

Clinically, ataxia and poor motor learning are the most common presentations and are nonprogressive compared with atrophic cerebellar disorders [46]. In infancy, hypotonia and global developmental delay are present earlier, and other signs include ocular motor disorders, dysarthria, intention tremor, and microcephaly. Behavioral abnormalities, intellectual disability, and speech and language disorders can vary from mild to severe impairment [26].

Management

It is important to consider that ataxia or other neurological signs in cerebellar hypoplastic patients usually do not worsen over time compared with atrophic cerebellar disorder. There is no standard course of treatment. The principal treatment is supportive including physical therapy, occupational therapy, speech therapy, psychiatric/behavioral medications, and special education.

Dandy–Walker Malformation

The fundamental structure that is affected in DWM is the cerebellum [47–49]. DWM is a genetic disorder, with the most common and severe type being the Dandy–Walker syndrome malformation [47]. The zinc finger protein of the cerebellum 1 (ZIC1) and zinc finger protein of the cerebellum 4 (ZIC4) genes on 3q24 [50] and FOXCI are candidate genes, and DWM results when they are deleted [51]. It is suggested that Zic1 and Zic4 are required for the full responsiveness of granule cell precursors (GCPs) to sonic hedgehog (SHH), but the role of the Foxc1 is not understood [36].

DWM is characterized by partial or complete agenesis of the vermis, upward rotation of the vermis, and an enlarged posterior cranial fossa [1, 36, 52, 53]. It is also characterized by cystic dilation of the fourth ventricle into the posterior cranial fossa. Enlargement of the posterior cranial fossa causes an abnormally high tentorium above the internal occipital protuberance and transverse occipital sulcus (location of transverse sinus) and also a variable degree of hydrocephalus [1, 54]. During cerebellar development, the right and left cerebellar primordia are fused at the midline. Any misregulation in this developmental process leads to a lack of cerebellar fusion at the midline. The lack of midline fusion causes the extension of membranous area/roof plate anteriorly, resulting in a large fourth ventricle. Cerebrospinal fluid pulsations cause roof plate expansion posteriorly within the posterior fossa, forming a large posterior cyst that represents the fourth ventricle [55].

Clinically, DWM can be defined via the characteristic triad consisting of the following: (1) complete or partial agenesis of the vermis, (2) cystic dilatation of the fourth ventricle, and (3) an enlarged posterior cranial fossa with upward displacement of the transverse sinuses [56, 57]. If hydrocephalous is present, it suggests a common developmental disorder in which multiple brain regions are affected [58].

The signs and symptoms associated with DWM are broad. DWM patients often have hypotonia, delayed motor development, ataxia, lack of coordination, jerky movements of the eyes, and progressive enlargement of the skull. Some patients may have normal cognition, whereas others have mild to severe mental retardation, even when hydrocephalus is effectively treated. The enlarged head circumference, which may bulge at the back of the skull, can increase pressure on the brain stem and nerves and can cause difficulties in controlling face and neck and abnormal

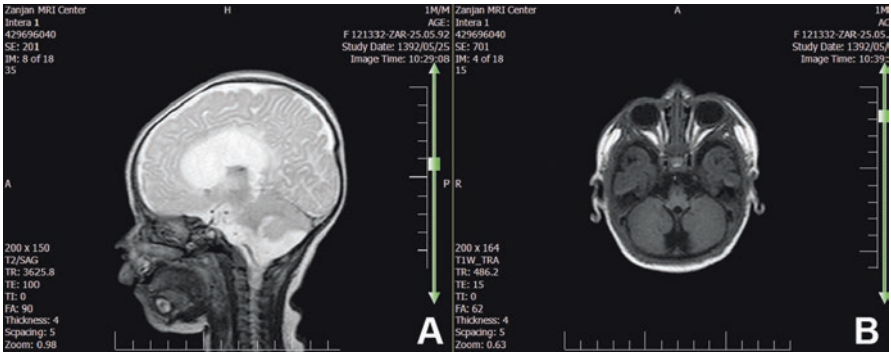

Fig. 2 Dandy–Walker malformation. (**a**) Sagittal T_2-weighted brain image. There is hypoplasia of the inferior vermis. A connection between the cisterna magna and the fourth ventricle is seen. (**b**) Axial T_1-weighted brain image. This image shows isolated inferior vermian hypoplasia and cerebellar hemispheres that appear normal. This is referred to as part of the Dandy–Walker variant

breathing patterns. Sagittal and axial MRI (Fig. 2) can distinguish DWM from other cerebellar malformations. In DWM, it is important to consider mega cisterna magna, retro-cerebellar cysts, and Blake's pouch cyst [55, 59]. It should be noted that in addition to the lack of the middle part of the cerebellum, the midline structures in the forebrain such as the corpus callosum may be absent. Systemic malformations associated with DWM may include cardiac anomalies, urogenital anomalies, and other abnormalities may occur collectively in about half of the patients [60–63].

Management

If there is hydrocephalous, treatment could include shunting and cerebrospinal fluid (CSF) drainage from the lateral ventricles and/or posterior fossa cyst, which is currently considered the surgical treatment of choice [49]. Treatment also consists of physiotherapy, occupational therapy, speech therapy, and specialized education. Although diagnosis of DWS during intrauterine development is difficult, if an ultrasound suggests DWS, then amniocentesis should be performed to aid in the diagnosis [64]. It is important that the families of affected children be referred for genetic counseling.

Joubert Syndrome and Related Disorders

JS was first identified by Marie Joubert in Montreal, Canada [65]. JS is a group of autosomal recessive conditions that are characterized by developmental anomalies, which are caused by defects in the structure or function of the primary cilium [66, 67].

JS consists of midbrain-hindbrain malformation, which causes a developmental delay, motor disability, hypotonia, ataxia, abnormal eye movements, and neonatal breathing abnormalities. One of the gold standards for JS diagnosis is the molar tooth sign (MTS) observed on a plain MRI (Fig. 3). When other organs, such as the retina, kidneys, and liver, are involved, it is called JS and related disorders (JSRDs), and these patients also have the MTS [66]. JSRDs are the most common inherited congenital cerebellar malformation.

Ciliopathy is a fundamental mechanism in JSRD. The primary cilia are important in neuronal development and function as cellular antenna that are found in nearly all cell types. The function of cilia in cells includes protein trafficking, photoreception, embryonic axis patterning, and cell cycle regulation. Therefore, dysfunction of this microtubule-based extension of cellular membranes can affect a single tissue or manifest as having multi-organ involvement, which is called ciliopathy [68]. Within the developing cerebellum, primary cilia have been shown to be essential for reception of the cell signaling ligand sonic hedgehog, which in turn is essential for proliferation of cerebellar neurons such as granule cells [69, 70].

The causative gene of many ciliopathies in individuals with JSRD has defined a new class of neurological diseases [68]. To date, over 16 causative genes have been associated with JSRD and all encode proteins in the primary cilium or its apparatus [66]. For example, mutations in genes such as AHII, INPP5E, CC2D2A, and ARL13B cause JS with MTS and retinal blindness [71]. However, mutations in TMEM216 and RPGRIPIL genes lead to MTS and renal involvement. In more severe cases, mutations in the CEP290 gene causes MTS together with retinal and renal involvement, while mutations in TMEM67 are the most common cause of MTS with liver involvement [72].

Clinically, JSRD patients have hypotonia, ataxia, dysregulated breathing rhythms (that result from dysfunction of the respiratory centers in the brainstem or cerebellum

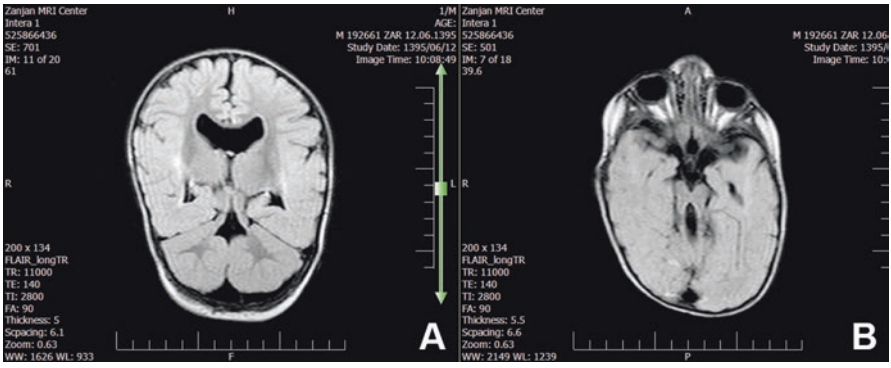

Fig. 3 Joubert syndrome and related disorders. (**a**) Coronal T_2 FLAIR brain image. The cerebellar vermis is aplastic and superior cerebellar peduncles are elongated. (**b**) Axial T_2 FLAIR brain image. This image shows a deep interpeduncular fossa, elongated superior cerebellar peduncles with cerebellar vermis hypoplasia, which are characteristic of the molar tooth sign in Joubert syndrome

[73]), abnormal eye and tongue movements, and subsequent mental retardation. As mentioned above, the key MRI finding in JSRD is MTS, which is the result of cerebellar vermis hypoplasia, and is accompanied by thick superior cerebellar peduncles with deep interpeduncular fossa. Because ciliopathy interrupts a broad range of developmental process, a defect could be seen in other organs such as kidney, retina, and liver, and there were also facial abnormalities (cleft lip or palate, tongue abnormalities) and polydactyly (extra fingers and toes) [74–76].

In mild JSRD, ataxic movement lessens with age and the ability to walk is delayed to age 4–5 years. Some neonates have died as a result of apnea, and therefore it is important to monitor neonates with JSRD during the first year of life. These patients should be periodically examined for any non-neurological signs and symptoms.

Management

The treatment is symptomatic and supportive such as physical therapy, occupational therapy, and speech therapy. Infants with abnormal breathing patterns should be monitored closely for apnea, and this may be required during the first year of life. In this case, caffeine may be helpful to promote respiratory drive. Because of the heterogeneity of these conditions, genetic testing will show specific gene mutations, which can help predict the range of organ involvement such as the retina, kidney, and liver [52].

Pontocerebellar Hypoplasia Type

PCH is a group of autosomal recessive neurodevelopmental and neurodegenerative disorders with hypoplasia of the cerebellum and ventral pons, followed by atrophy. It is also characterized by variable cerebral involvement such as microcephaly, seizures, and a severe delay in cognitive and motor development, which in many cases is fatal early in life [77–79].

Ten different subtypes have been reported based on clinical and genetic features (i.e., PCH1-10) [80], and they are summarized in Table 1. Mutations in the following genes cause PCH because of molecular malfunctions that are important for normal development of the neurons and nonneuronal cells. Mutations in the VRK1 gene on chromosome 14q32.2 cause PCH IA (or spinal muscular atrophy with pontocerebellar hypoplasia; SMA-PCH), in which there is spinal cord anterior horn cell degeneration [81, 82]. Mutations in the EXOSC3 gene on chromosome 9p13.2 lead to PCH1B [83]. Mutations in three genes, TSEN2, TSEN34, and TSEN54, encoding three of four subunits of the tRNA splicing endonuclease complex have been found to underlie PCH2, PCH4, and PCH5 [77]. PCH2 is characterized by cerebellar hypoplasia in which the hemispheres are more severely affected than the vermis,

Table 1 Types of PCH

Gene	Chromosome	PCH types
VRK1	14q32.2	PCH IA
EXOSC3	9p13.2	PCH1B
TSEN34	17q25.1	PCH2A
TSEN2	3p25.2	PCH2B
TSEN34	19q13.42	PCH2C
SEPSECS	4p15.2	PCH2D
VPS53	17p13.3	PCH2E
TSEN15	1q25	PCH2F
PCLO	7q21	PCH3
TSEN54	17q25.1	PCH4
TSEN54	17q25.1	PCH5
RARS2	6q15	PCH6
?	**?**	PCH7
CHMP1A	16q24	PCH8
AMPD2	1p13	PCH9
CLP1	11p12	PCH10

and in contrast to PCH I, there is no anterior horn cell degeneration in the spinal cord. These patients have other signs and symptoms such as progressive cerebral atrophy, microcephaly, dyskinesia, and seizures [77, 78]. Gene mutations affect PCH1-10, as follows: mutations in TSEN54 on chromosome 17q25.1 cause PCH2A; mutations inTSEN2 on chromosome 3p25.2 cause PCH2B; mutations in TSEN34 on chromosome 19q13.42 cause PCH2C; mutations in SEPSECS on chromosome 4p15.2 cause PCH2D (as known as progressive cerebello-cerebral atrophy; PCCA); mutations in VPS53 on chromosome 17p13.3 cause PCH2E; and mutations in TSEN15 on chromosome 1q25 cause PCH2F [84–86]. PCH3 is caused by a mutation in the PCLO gene on chromosome 7q21 [87], and PCH4 is caused by a mutation in the TSEN54 gene on chromosome 17q25.1 [84]. A mutation in the TSEN54 gene on chromosome 17q25 causes PCH5, and a mutation in the gene encoding mitochondrial arginyl-tRNA synthetase (RARS2) on chromosome 6q15 causes PCH6 [88]. The gene involved in PCH7 is unknown [89, 90]. PCH8 is caused by a mutation in the CHMP1A gene on chromosome 16q24 [91]. A mutation in the AMPD2 gene on chromosome 1p13 causes PCH9 [92] and a mutation in CLP1 on chromosome 11p12 causes PCH10 [93]. Finally, loss of function of SLC25A46 causes lethal congenital pontocerebellar hypoplasia [94].

Clinically, PCH patients have hypotonia and difficulty with coordination of sucking and swallowing and problems handling their oral and respiratory secretions [95]. There are no criteria to distinguish precisely between the different subtypes based on clinical signs and symptoms, and therefore genetic testing is important. The cerebellum and pontine hypoplasia can be revealed by MRI in which the cerebellar hemispheres may be more severely affected than the midline vermis (Fig. 4).

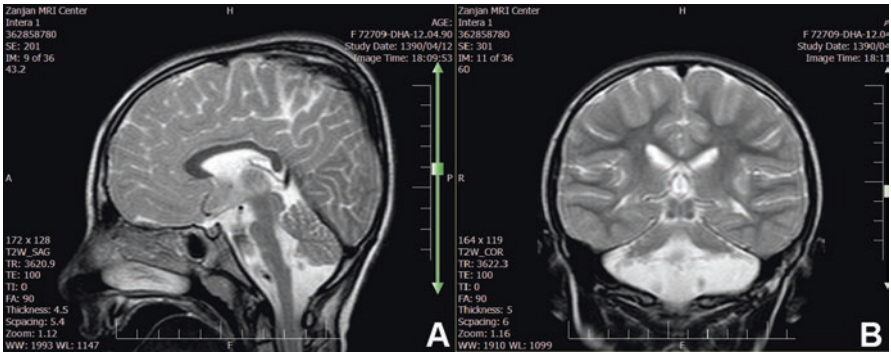

Fig. 4 Pontocerebellar hypoplasia. (**a**) Sagittal T_2-weighted brain images. Sagittal image: the pons is very small but has a relative sparring bulging in its superior part. Vermis hypoplasia predominates at the inferior site. (**b**) Coronal T_2-weighted brain images. Cerebellar hemispheric hypoplasia with vermis relatively spared leading to classic dragonfly image

Management

Treatment is symptomatic and requires the teamwork of health-care professionals. Patients with PCH need a gastrostomy tube and airway control, and they may not survive beyond 1 year of age. There is no known cure for PCH. It is important to refer families of affected children for genetic counseling.

Rhombencephalosynapsis

RES is a neurodevelopmental malformation that is characterized by midline fusion of the two cerebellar hemispheres, which is caused by failure of the midline structure development in the rhombencephalon. It is suggested that disruption of dorsoventral patterning of the rhombencephalon may cause RES [96]. RES is rare condition with unknown etiology, and the most specific and key MRI finding is agenesis or hypogenesis of the vermis, in which the cerebellar vermis is completely or partially absent with a fused cerebellar hemisphere and midline dentate nucleus [96]. RES may be associated with other cerebellar abnormalities, such as Purkinje cell heterotopias [97]. RES can be seen as an isolated cerebellar disorder or together with other developmental malformations in the nervous system or other organs. Although RES is seen most frequently in isolated form, it is a highly consistent finding in Gomez–Lopez–Hernandez syndrome (GLHS). GLHS is also known as cerebellotrigeminal-dermal dysplasia (a neurocutaneous disorder). Isolated RES malformation should be distinguished from cerebellotrigeminal-dermal dysplasia, which presents with parietal/temporal alopecia (lack of hair), trigeminal anesthesia (loss of sensation in the face), midface hypoplasia with towering skull shape, corneal opacities, mental retardation, and short stature. RES is also associated with

midline brain structural defects including absent olfactory bulbs, dysgenesis of the corpus callosum, absent septum pellucidum, and in rare patients, atypical forms of holoprosencephaly [96]. RES has also been reported in vertebral anomalies, anal atresia, cardiovascular anomalies, trachea–esophageal fistula, renal anomalies, limb defects (VACTERL) association, and hydrocephalus [26, 97–100].

Ishak et al. [96] proposed four groups based on the severity of cerebellar vermis defect: (1) mild, in which the nodulus, anterior, and posterior vermis are partially absent; (2) moderate, where there is a lack of posterior vermis with some anterior vermis but the nodulus is present; (3) severe, which is a lack of posterior and anterior vermis with the nodulus partially absent; and (4) complete, where there is a lack of the entire vermis [96]. They also divided RES-affected patients into four clinical categories using the following criteria: (1) RES in patients with GLHS, (2) RES plus at least one of the VACTERL features without scalp alopecia, (3) RES plus a focal or diffuse forebrain midline fusion defect without alopecia, and (4) RES in patients with malformations that do not fit into the categories 1–3 (with abnormal head shape, midface hypoplasia, low-set and/or posteriorly rotated ears, telecanthus, and/or hypertelorism).

Clinically, signs and symptoms in patients with the isolated form of RES are variable including developmental delay, in which motor learning and skills develop between 3 and 6 years of age, hypotonia, and ataxia [101].

Management

Treatment for RES infants is generally supportive and includes physical therapy and occupational therapy. If hydrocephalus is present in patients with RES and it is symptomatic, this can be an indication for surgical intervention with a ventriculostomy or ventricular shunt. It is important to refer families of affected children for genetic counseling.

Lissencephaly and Cerebellar Dysplasia

Lissencephaly with cerebellar hypoplasia is a neurodevelopmental malformation in which cellular migration is severely impaired. The cerebellum in patients with lissencephaly is underdeveloped with prominent vermis hypoplasia or aplasia [102–105]. Mutations in the gene encoding reelin (RELN), which is mapped on chromosome 7q22, cause lissencephaly with severe abnormalities of the cerebellum, hippocampus, and brainstem. Reelin is a large extracellular matrix-associated protein [106] that is involved in migration of neurons through binding to its receptors (very low density lipoprotein receptor [VLDLR]), the apolipoprotein E receptor 2 (ApoER2; [107–109]), and also α3β1 integrin and protocadherins [110]. In a mouse model of lissencephaly, mutations in RELN and DAB1 prominently cause

neuronal migration defects in the brain with accompanying cerebellar hypoplasia, and there is also abnormal circuitry development [111, 112]. Mutations in reelin show abnormal developmental disorders outside the brain as well such as neuromuscular connectivity and congenital lymphedema [104]. It is also reported that mutations in α-dystroglycan may result in lissencephaly and central nervous system developmental malformations [113].

Clinically, the important approach is magnetic resonance imaging (MRI) from the cerebellum, which shows severe vermis and cerebellar hypoplasia and cerebellar peduncle malformation.

Management

Treatment of patients who have lissencephaly with cerebellar hypoplasia is supportive care and treatment of symptoms. In case of difficulties with feeding, a gastrostomy tube may be considered. If seizures are present, anti-seizure medications are administered, and in the case of hydrocephalus, shunting is performed. It is important to refer families of affected children for genetic counseling.

Dysplastic Cerebellar Gangliocytoma or Lhermitte–Duclos Disease

The first case of the LDD was reported by Lhermitte and Duclos in 1920 as a cerebellar ganglion cell tumor or dysplastic cerebellar gangliocytoma [114, 115]. LDD is a rare developmental disorder of the cerebellum and features both malformation and benign neoplasm. Most patients with LDD appear to have mutations in the phosphatase and tensin homologue (PTEN) gene [115–117]. Most frequently, LDD occurs in young adults in the third and fourth decades of life [118, 119]. Because LDD presents in previously healthy children with features of a unilateral cerebellar mass, the main considerations are the posterior fossa tumor and secondary hydrocephalus. LDD is not diagnosed as medulloblastoma in most patients because of differences in the age group, medical history, and unique imaging features. Neuroimaging with MRI is sufficient and important in the diagnostic process. Longstanding unilateral space-occupying skull lesions in the posterior fossa leads to thinning of the skull in the occipital region [120, 121]. Histopathological findings show dysplastic gangliocytoma of the cerebellum in front of a hamartoma lesion with widening of the molecular layer occupied by abnormal ganglion cells, absence of the Purkinje cell layer, and hypertrophy of the granular layer [122].

Clinically, patients with LDD present with headache, nausea, cerebellar signs, hydrocephalus, and increased intracranial pressure. Patients may have symptoms for many years, such as cranial nerve palsies and cerebellar symptoms, because of

the slowly progressive nature of this disease [120]. LDD patients may show mental retardation. LLD is commonly associated with other congenital malformations such as familial hamartoma-neoplasia syndrome and Cowden's disease (CD), an inherited cancer/hamartoma syndrome involving the breast, thyroid gland, and other organs [123].

Management

Decompressive surgery for symptomatic patients is the accepted choice of treatment. The risk of performing surgery is the lack of clear tumor margins. Symptomatic and supportive treatments such as physical therapy and occupational therapy should be offered.

Summary

In this chapter, we summarized cerebellar malformations and current treatment. Treatment is in response to symptoms and requires a team of specialists, health-care professionals, and genetic counselors. Based on available knowledge and our experience, there is no curative treatment and most of the patients are managed using conservative approaches.

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Clinical Features, Assessment, and Management of Patients with Developmental and Other Cerebellar Disorders

Michael S. Salman

Abstract The cerebellum is essential for processing, modulating, and controlling movement, behavior, and cognitive functions. Cerebellar disorders cause tremor and incoordination, larger variability, and inaccuracy of movements during eye and limb movements, stance, and speech. Cerebellar dysfunction also results in impaired cognition and behavior. During the clinical assessment, details of the presenting complaints including onset and time course of ataxia, other symptoms, past medical history including developmental milestones, family history, and drug history are elicited. On examination emphasis is placed on examining the motor system especially speech and eye and limb movements. Other aspects include general examination, head size, dysmorphic features, neurocutaneous stigmata, and cognitive function assessment. A thorough exam of the cranial nerves, tone, strength, coordination, reflexes, gait, and sensation should be undertaken. A comprehensive assessment helps to narrow down the diagnostic possibilities and offers clues to specific disorders of the cerebellum. Management is guided by disease etiology.

Keywords Cerebellum • Motor coordination • Eye movements • Speech articulation • Cognitive function

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Introduction

Ataxia is a relatively common presentation in the pediatric population with an estimated prevalence rate of 26 per 100,000 children in Europe. The annual crude incidence rate of chronic ataxia is 3.2 per 100,000 children and adolescents residing in Manitoba, Canada. Ataxia is caused by numerous diseases [1–5].

This chapter discusses the clinical features in children with cerebellar disorders including motor abnormalities, cognitive, affect, and behavioral dysfunction. The clinical assessment of patients with developmental and other cerebellar disorders is described, and different aspects are discussed in detail.

Many clinical motor features of cerebellar disease and their interpretation have been described succinctly by Dr. Gordon Holmes in his Croonian lectures in 1922 [6]. New roles for the cerebellum in health and disease continue to emerge with evidence implicating Purkinje cell dysfunction in the latter [7]. More recently, few comprehensive reviews and consensus papers on symptoms and signs of cerebellar dysfunction, roles of the cerebellum in motor control, and nonmotor role of the cerebellum in language and other related disorders have been published [8–11].

Limbs' Motor Control

Smooth and accurate execution of voluntary movements and adaptation to changing demands of motor tasks rely on an intact cerebellum [12]. The cerebellum can learn and store different combinations needed for precise complex movements through trial and error. Patients with cerebellar lesions can perform simple motor tasks. However, incoordination and impaired initiation of movement appear when compound complex movements are performed especially at a fast pace [13]. Cerebellar dysfunction causes greater impairment in predictive movements than in movements requiring feedback, for example, visual or somatosensory feedback [14]. Patients with cerebellar disorders appear to have proprioceptive deficits during active but not passive limb movements [15]. Furthermore, the ability to adapt to novel changes in movements is impaired. Table 1 shows several clinical motor signs in patients with cerebellar disease.

Ocular Motor Control

The cerebellum serves an important part for the normal functioning of all types of eye movements including saccades, smooth ocular pursuit, modulation of the vestibulo-ocular reflex, and also for ensuring visual fixation stability. The cerebellum fine-tunes eye movements and reduces their baseline variability to ensure that the two eyes are stable and working together. This is essential for bringing and maintaining objects of interest on or very close to the fovea. This, in turn, leads to

Table 1 Cerebellar signs causing abnormal control of stance and voluntary movements

Sign	Comment
Asthenia	Delay in initiating muscle contraction and slow attainment of full force. It can be elicited by asking the patient to grasp the examiner's hand firmly
Adventitiousness (inappropriate accessary movements)	Failure to fix the proximal muscles to preserve the correct posture in relation to the moving part of a limb. This represents exaggerated activation of muscles that should be paused
Dysdiadochokinesia	Slowness and irregularity of the frequency and amplitude of rapid alternating movements. It can be observed during successive pronation and supination of the forearm at the elbow joint. It also manifests with difficulty on repeating the syllables pa-ta-ka
Rebound	Abnormally large displacement of an outstretched arm following a tap on the wrist with overshooting followed by few oscillations around the primary position
Dysmetria	Inaccurate movement trajectory with under- or overshooting a target. It can be observed during finger-nose exam or heel-to-shin exam. It is speed and inertia sensitive
Intention tremor	Oscillation of a limb especially when approaching a target during goal-directed voluntary movements. It can be observed during finger-nose exam or heel-to-shin exam
Kinetic tremor	Oscillation of a limb at the commencement of voluntary movements
Postural tremor	Oscillations observed during postural tasks, e.g., maintaining the heel of one foot over the contralateral knee for a few seconds or maintaining the outstretched arms parallel to the ground. It affects proximal > distal muscles
Palatal tremor	Rhythmic oscillations of the palate
Titubation	Involuntary rhythmic oscillations of a body part, e.g., the head or trunk
Head tilt	Lateral displacement of the head
Truncal ataxia	Swaying of an unsupported sitting or standing trunk
Ataxia of stance	Swaying of the body while standing up
Ataxia of gait	Wide-based gait with staggering and swaying. Tandem gait and running unmask more subtle gait ataxia
Inability to perform the Romberg maneuver with the eyes open	Inability to stand with the legs and feet touching each other while the eyes are open
Dysrhythmokinesia	Abnormal rhythm observed during tapping of a limb
Abnormal handwriting or drawing	A written sentence will appear irregular, large, and tremulous. An Archimedes' spiral will appear tremulous and dysmetric
Hypotonia	Decreased resistance to passive stretch
Pendular reflexes	Excessive oscillations of a limb (like the swing of a pendulum) observed after eliciting a deep tendon jerk
Motor delay	Slow acquisition of motor milestones

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the best visual acuity whether the person is moving or not [8]. Three cerebellar regions are important for ocular motor control: the flocculus/paraflocculus, the nodulus-ventral uvula, and the dorsal oculomotor vermis/fastigial oculomotor region [8, 16].

Various types of nonphysiological nystagmus (i.e., pathological ocular oscillations), for example, gaze-evoked nystagmus and saccadic intrusions (abnormal fast eye movements that take the fovea off the target), occur following cerebellar damage and result in fixation instability [9, 16]. Saccadic (jerky) smooth ocular pursuit and saccadic dysmetria (hypo- or hypermetria) are other well-recognized ocular motor signs of cerebellar dysfunction [16]. Table 2 shows several ocular motor signs in patients with cerebellar disease.

Speech Control

The production of speech is a complex process that involves several neural networks located in the cerebrum and cerebellum [10]. The production of speech involves the coordination of a large number of muscles, in particular the tongue and orofacial muscles [17]. The cerebellum plays an important role in speech articulation, prosody (i.e., characteristics of speech style including speed, rhythm, pitch, and emphasis), and planning and processing of speech and language [18].

Cerebellar impairment can cause ataxic dysarthria [10]. Abnormalities in speech motor programming through impaired timing and deficits in speech execution are both implicated in ataxic dysarthria [18]. Table 3 shows key features of speech abnormalities in patients with cerebellar disorders.

Nonmotor Impairments in Cerebellar Disorders

A multitude of studies support nonmotor roles for the cerebellum in cognition and behavior control. Cerebellar abnormalities have been identified in patients with cognitive and neuropsychiatric disorders. In addition, developmental delay, learning difficulties, and behavioral problems have been commonly reported in children with developmental cerebellar disorders [11].

Language

The cerebellum modulates several aspects of language production and perception [8]. In addition, the cerebellum is involved in reading and writing [10]. Cerebellar impairment results in disturbances in syntax processing, prosody, and grammar [19], with anomia, perseveration, and reduced speech output and speed [20, 21].

Table 2 Cerebellar ocular motor signs

Sign	Comment
Gaze-evoked nystagmus	Ocular oscillations observed while trying to hold gaze eccentrically (i.e., off-center), horizontally, and/or vertically. The fast phase of the nystagmus is toward the direction of gaze
Downbeat nystagmus	Ocular oscillations observed with the eyes in central position (i.e., the eyes are located in the primary mid-orbital position). The fast component beats downward. The nystagmus is exacerbated in downgaze and lateral gaze
Upbeat nystagmus	Ocular oscillations observed with the eyes in central position. The fast component beats upward. The nystagmus is exacerbated in up gaze
Rebound nystagmus	Transient ocular oscillations observed with the eyes in central position after returning from a maintained eccentric gaze
Periodic alternating nystagmus	Horizontal ocular oscillations observed with the eyes in central position that change direction gradually after a silent phase. It occurs in a periodical manner, usually every 1–2 min
Opsoclonus	Conjugate, random, involuntary, and multidirectional back-to-back fast eye movements observed during attempted fixation or movement of the eyes
Ocular flutter	Conjugate, random, involuntary, and horizontal back-to-back fast eye movements observed during attempted fixation or movement of the eyes
Ocular bobbing	Fast downward displacement of the eyes followed by slow return back to the central orbital position
Square wave jerks/ macro-saccadic oscillations	Fast, intruding, unwanted, involuntary, and conjugate eye movements, which take the eyes off fixation. They may occur repetitively
Saccadic dysmetria	Inaccurate fast eye movement that either undershoot (hypometria) or overshoot (hypermetria) a visual target
Saccade initiation delay (previously misnamed as ocular motor apraxia)	Increased latency of fast eye movements that can usually be overcome with a head thrust or a blink
Slowing of smooth pursuit velocity (especially initiation)	Jerky (instead of smooth) eye movements that are observed during visual tracking
Impaired response of the vestibulo-ocular reflex	The vestibulo-ocular reflex normally drives the eyes contralateral to the direction of the head movement. Abnormal amplitude and direction of eye movements during the head impulse test may occur in cerebellar disease. The patient is asked to fixate on the examiner's nose, while the head is actively and briskly rotated about 15° to the right and left
Impaired vestibulo-ocular reflex cancellation (VORc)	The ability to fixate objects moving in the same direction of the head requires cancellation of the vestibulo-ocular reflex. Patients with cerebellar disease may not be able to cancel the vestibulo-ocular reflex

(continued)

Table 2 (continued)

Sign	Comment
Abnormal optokinetic nystagmus	Fast ocular oscillations (jerk nystagmus) are normally observed while tracking a rotating drum with alternating white and black stripes. The nystagmus generated with such a stimulus may be exaggerated with chronic cerebellar disease or dampened with acute cerebellar lesions
Impaired adaptation of eye movements	Motor learning (adaptation) of the ocular motor system usually occur physiologically or following disease to repair and improve the accuracy or velocity of eye movements. Adaptation may be impaired in cerebellar disease
Skew deviation	Non-paralytic vertical misalignment of the eyes (i.e., one eye is higher than the fellow eye) which changes as a function of horizontal gaze position
Esotropia	Non-paralytic horizontal misalignment of the eyes with inward deviation
Abnormalities in the control of torsion	Abnormal rotational control of the eye around an axis perpendicular to the center of the pupil

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Table 3 Speech abnormalities in cerebellar diseases

Scanning speech (e.g., hesitation, accentuation of some syllables, omission of appropriate pauses, addition of inappropriate pauses)
Explosive speech
Slowness of speech
Syllables or words are not understandable with lack in speech clarity
Slurring of speech
Loss of intonation (abnormal rhythm and emphasis)
Voice tremor

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Cognition

Investigations on the cerebellar contribution to cognition are consistent with a role for the lateral cerebellar hemispheres in supporting cognitive processes [22]. In children, significant cognitive disruption is associated with pediatric cerebellar diseases ranging from cerebellar developmental abnormalities to inflammatory disorders, ischemic injury, oncologic, and postsurgical injury [23–32]. These cognitive deficits are associated with executive dysfunction, impairment in working memory, procedural memory, and processing abilities, in addition to a lower intellectual quotient and visuospatial abilities.

Affect and Behavior

The cerebellum is thought to modulate behavior. Schmahmann described the cerebellar cognitive-affective syndrome, which manifests with significant behavioral difficulties in patients with cerebellar disorders. The author and his colleagues described behaviors ranging from affective changes to disinhibited behaviors [19]. Other investigations of cerebellar lesions have supported these initial descriptions with many associated behavioral difficulties including alterations in attention, affective disruption, emotional and social blunting, anxious behaviors, and obsessive and compulsive behaviors [19, 27, 33, 34].

Assessment of Pediatric Patients with Developmental and Other Cerebellar Disorders

History

The assessment of patients with pediatric cerebellar disorders starts with a detailed clinical history, which can lead to the diagnosis in as many as 80% of patients [35]. Details of the presenting illness and complaints should be elicited including the age and date of onset, mode of the ataxia onset (i.e., acute, subacute, or chronic), location including whether the symptoms are unilateral or bilateral, severity, duration, rate of progression, factors that make the symptoms better or worse, possible triggers, and medications used [3–5, 36]. An inquiry should be specifically made about the presence of vertigo, dizziness, imbalance, oscillopsia, and blurred vision [8]. Systematic inquiry into other symptoms should then be pursued [35], including headache, confusion, developmental regression, seizures, numbness, tingling, and weakness.

Age of the parents at conception, previous miscarriages, mother's health and toxin exposure during pregnancy, antenatal screening and problems during pregnancy, birth history, birth weight, length, and head circumference, early feeding or respiratory difficulties, neonatal course, the number of days spent in hospital after birth, and past medical history are important part of the assessment.

Observing videos of children at different ages can be very valuable [36]. Developmental milestones may give further clues. For example, many patients with nonprogressive ataxia without brain malformations or with developmental cerebellar disorders manifest with motor delay and hypotonia before the ataxia becomes apparent [37–40].

Drug history and possible exposure to toxins or drugs should be obtained [4, 35]. Ethnicity, family history of consanguinity, ataxia, or other symptoms and disorders may all offer useful diagnostic clues [5]. However, it is important to be aware of challenges when obtaining the family history [41]:

1. Young parents or grandparents in autosomal dominant disorders (age-dependent penetrance). In such situation, the disease may not have manifested in family members yet.
2. Incomplete penetrance. The disease is not apparent in affected family members.
3. Early death in carriers from an unrelated cause.
4. New (de novo) mutations.
5. Lack of awareness of disease in family members especially further than one or two generations (i.e., the disorder is not known in the past or is unrecognized, or if the individual affected has not sought an assessment, or information on deceased relatives is not passed on).
6. Hidden or concealed symptoms from family members.
7. Family members may be divorced or scattered or had symptoms after they are out of touch.
8. Nonpaternity, infertility, adoption, or egg/sperm donation.
9. Small family with no affected members.
10. Negative prior genetic testing. It is important to inquire about what test was done, when, and how. New advances in techniques may have occurred since the test was done, pathogenicity of variants of unknown significance has been found, a previously unknown abnormality has been reported, or a newly described disease has been published.

Physical Examination

Careful general and then more focused examination should then be undertaken to look for cerebellar (Tables 1, 2, and 3) and non-cerebellar signs [4, 5, 35, 39, 40], for example, head size; weight; height; dysmorphic features; neurocutaneous stigmata (i.e., skin abnormalities that may be indicative of an underlying brain malformation); other skin lesions, e.g., telangiectasia; respiratory, cardiac, and abdominal examination for enlarged liver and spleen; scoliosis; pes cavus; contractures; and wasting.

Visual acuity, visual fields, pupillary reaction to light and near objects, and funduscopy exam in each eye should be done. A careful assessment of the different classes of eye movements in patients with ataxia can be quite helpful and may offer clues to the diagnosis [42]. A practical and comprehensive guide on the examination and interpretation of eye movements in children is available for the interested reader [43]. Ocular alignment, fixation stability, slow and fast eye movements (including smooth ocular pursuit, convergence, vestibulo-ocular reflex and its cancellation, and saccades) (Table 2) should be ascertained. In addition, facial and tongue movements, bulbar (ability to swallow liquid and solid food safely and without choking), speech (voice quality, clarity, prosody) (Table 3), tone (resistance to passive stretch), strength, coordination of the upper and lower limbs, reflexes, plantar response, various sensation modalities including proprioception, and gait should then be assessed (Table 1) [16].

Table 4 Cognitive and behavioral abnormalities in cerebellar diseases

Language (nonmotor speech, reading, writing)
Executive function and working memory
Autistic behavior (repetitive/restricted, social impairment)
Attention deficit hyperactivity disorder
Schizophrenia
Anxiety behavior
Mood disorders

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In young infants and toddlers, an opportunistic approach is recommended, at least initially, as the child may not be fully cooperative. A lot of information can be gleaned by hearing the child talk and watching the child interact with the parents, other siblings, or the physician during history taking. In addition, watching the child play, use an iPad, or move around the clinic room can be invaluable. It is worth paying attention to the child's affect, behavior, language use, and cognitive abilities. Are there any features suggestive of the cerebellar cognitive-affective syndrome (some of these features are shown in Table 4)?

Extracerebellar features should be looked for to identify red flags [4, 35]. For example, swollen optic disks suggest an expanding mass; decreased visual acuity from optic neuritis suggests acute disseminated encephalomyelitis or multiple sclerosis; altered level of consciousness suggests acute disseminated encephalomyelitis, stroke, or intoxication; facial nerve palsy, hearing loss, tinnitus, nausea, and vomiting may indicate brainstem compression from a tumor; apraxia of gait may be caused by hydrocephalus or Rett syndrome; and head size, if large then hydrocephalus should be excluded, and if small then genetic, viral, or metabolic diseases that affect the cerebrum should be pursued. Pyramidal tract signs (spasticity, hyperreflexia, Babinski's sign, or clonus), seizures, and dyskinesia imply involvement of the cerebrum.

Pitfalls in the Assessment of Ataxia

Although disorders of the cerebellum and its input or output tracts can cause incoordination, which we refer to as ataxia, it is important to exclude mimickers of ataxia, i.e., pseudoataxia. Poor coordination may result from many causes including decreased level of consciousness; subtle seizures; postictal state; nonconvulsive status epilepticus; extrapyramidal movement disorder; spasticity; weakness, e.g., from peripheral neuropathy; clumsiness only (i.e., developmental coordination disorder); muscular or skeletal disorders, for example, irritable hip; and psychogenic disorders [35, 44].

Formulating a Clinical Impression and a Plan of Investigations

After the history and physical examination are completed, the pattern of abnormalities is summarized. Variations in the clinical phenotype in relation to several disease etiologies in 184 children with chronic ataxia have been recently explored using latent class analysis. Few specific clinical patterns emerged that were highly associated with certain disease etiologies [45]. For example, if a child presents with global developmental delay, hypotonia, and seizures (which may occur before the ataxia becomes manifest), then Angelman syndrome, disorders of neuronal migration, and Joubert syndrome and related disorders should be suspected. A brain MRI with thin cuts will likely show the neuronal migration abnormalities, while genetic testing is needed for the diagnosis of Angelman syndrome where brain MRI is typically normal. In addition Joubert syndrome and related disorders have diagnostic MRI features known as the molar tooth sign. Another example is the child that has no history of seizures and has symptoms onset including ataxia at greater than 10 years of age with otherwise normal development but has slurred or scanning speech. In such a clinical scenario, episodic ataxia and Friedreich ataxia should be considered. If the ataxia is progressive, then Friedreich ataxia should be suspected first, but if the symptoms are intermittent, then episodic ataxia should be considered first. This clinical approach may aid the diagnostic process by making it more efficient. In general, one should ascertain the following:

1. What regions/networks are affected by the incoordination? Specifically, head, eye movements, speech, swallowing, arms, and gait involvement should be documented. There is a rough map for localizing cerebellar symptoms and signs. For example, symptoms of damage of the lateral cerebellar hemisphere include hypotonia, asthenia, intention tremor, and dysmetria, while vermal and paravermal lesions are associated with ataxia of gait and stance. Similarly, damage to the dorsal vermis and fastigial nuclei is associated with saccadic dysmetria and impaired saccadic adaptation, while damage to the vestibulocerebellum is associated with impaired smooth ocular pursuit and various types of nystagmus [8, 16, 46].
2. What is the mode of ataxia onset? Acute onset is suggestive of toxic, metabolic, vascular, or traumatic etiologies. Subacute onset may indicate infectious, inflammatory, or paraneoplastic etiologies, while chronic ataxia is more likely to be caused by genetic or neurodegenerative disorders [41]. There is however an overlap in the mode of onset among the different etiologies.
3. How does the ataxia change over time? Is the ataxia improving thus suggesting a postinfectious etiology, nonprogressive suggesting a cerebellar malformation, recurrent (i.e., episodic or intermittent with resolution between the episodes) suggesting an episodic ataxia or a metabolic disorder, or is the ataxia progressive suggesting a tumor or a neurodegenerative disorder? This information will help focus the investigations on more likely etiologies [3–5, 35, 36, 41].
4. Is it pure ataxia? Some diseases only affect the cerebellum, thus narrowing the list of diagnostic possibilities.

5. Are there any clues in the family history?
6. Are there non-ataxia central nervous system features? For example, spasticity, dyskinesia, seizures, or optic atrophy implies widespread central nervous system involvement beyond posterior fossa structures [41].
7. Are other organs affected? For example, heart, liver, and kidney involvement raises suspicion of a metabolic disorder.

Based on the clinical impression, a plan of investigation is carried out [3, 35, 41, 45]. Neuroimaging is usually very helpful, even when it is normal [44]. A brain magnetic resonance imaging (MRI), with magnetic resonance angiography and spectroscopy if indicated, offers the best spatial resolution of cerebellar and extracerebellar brain structures [44, 47]. A spinal MRI is occasionally helpful. For example, it may reveal spinal cord atrophy in patients with Friedreich ataxia [44]. In selected patients repeating a brain MRI several months or few years after the first brain MRI may offer further diagnostic clues in patients, who remain without a diagnosis despite extensive investigations [44].

Biochemical tests, drugs and toxins screen, and metabolic investigations on blood, urine, or where appropriate cerebrospinal fluid are then performed in a stepwise manner [3, 5, 41]. These include but are not limited to the following: full blood count, ESR, CRP, glucose, electrolytes, calcium, magnesium, phosphorus, albumin, creatinine kinase, liver and thyroid function tests, cholesterol, alpha fetoprotein, immunoglobulins, autoimmune antibodies (including ANA, ANCA, antigliadin antibodies), and metabolic tests (including ammonia, lactate, amino acids, ceruloplasmin, transferrin isoelectric focussing, uric acid, total and free carnitine, acylcarnitine, very long chain fatty acids, lysosomal enzymes, vitamins E, B1, and B12, phytanic acid, urine organic acids and amino acids, and CSF neurotransmitters).

Many genetic tests are available [36, 47], and are usually also requested in a stepwise manner guided by findings from the clinical assessment and neuroimaging findings. The tests include microarray, karyotype, FISH, calcium channel mutations, and mutations in selected spinocerebellar ataxia genes. Gene panel testing is another option for diseases with similar phenotypes. Whole-exome sequencing is not widely available in routine clinical practice but is proving to be a useful investigation in patients with undiagnosed ataxia.

Nerve conduction studies, electromyogram, electroencephalogram, and skin and muscle biopsies may also be indicated in some patients with ataxia.

Management

Management of the patients starts with discussing the findings of the clinical assessment with the patient and their parents. The discussion needs to be done honestly and in a sensitive manner. Every effort should be made to avoid using technical and medical jargons, taking the age of the patient and level of parental education into account. Diagnostic uncertainties and limitations should be disclosed. A plausible

list of diagnostic possibilities or details on a specific disorder when a diagnosis is made should then be discussed. Prognosis and availability of antenatal diagnosis for families that are interested in having more children should be mentioned. Referral to a geneticist for further investigations and counseling should be made, if indicated.

Treatment of the underlying disease etiology in acquired ataxias is possible in some disorders, for example, tumors, strokes, avoidance of toxins and certain medications, and inflammatory disorders [3].

General nonspecific management options for the symptomatic treatment of ataxia include physiotherapy, occupational therapy, and referral to other rehabilitation specialists. Continuous intensive motor training is beneficial [48]. Drugs and noninvasive cerebellar stimulation techniques such as transcranial magnetic stimulation or transcranial direct current stimulation are being explored, and preliminary studies show possible therapeutic benefit [48]. Referral to a speech and language pathologist in patients with dysarthria and speech or language delay should be made. Social workers and referral to support organizations such as the National Ataxia Foundation can be invaluable to the patients and their families [41].

There are limited treatment options available for the ataxic patients. Treatments for developmental cerebellar disorders and most hereditary ataxias are generally not available, and specific treatments are only available for a handful of diseases that are usually caused by metabolic dysfunction [3, 5, 48]. For example, vitamin E is given to patients with abetalipoproteinemia or ataxia with vitamin E deficiency, biotin to patients with biotinidase deficiency, coenzyme Q to patients with coenzyme Q deficiency, acetazolamide or 4-aminopyridine to patients with episodic ataxia type 2, nicotinamide for Hartnup disease, dietary modification and thiamine to patients with maple syrup urine disease, dietary modification and sodium benzoate to patients with urea cycle defects, and the ketogenic diet to patients with pyruvate dehydrogenase deficiency.

Other symptoms associated with ataxia should also be addressed and treated, for example, epilepsy, spasticity, sleep disturbance, behavioral difficulties, and anxiety.

Patients with multisystem disease should be referred to other specialists [41]. For example, patients with Friedreich ataxia should be referred to an endocrinologist, as they are at risk of developing glucose intolerance and diabetes, and a cardiologist since a life-threatening cardiomyopathy can occur in this disorder where possible treatments are available including idebenone, vitamin E, and coenzyme Q.

Conclusions

The cerebellum functions beyond motor coordination (Table 4). Roles for the cerebellum in children are identified in motor functions, cognition, and behavior in both normal development and also in disease. Since a significant part of cerebellar development stretches from the third trimester of pregnancy to the early postnatal years, diverse causes of cerebellar disruption contribute to the pathogenesis of

neurodevelopmental disorders. A comprehensive detailed history and physical examination are essential components of the clinical assessment in patients with cerebellar diseases and usually guide clinical investigations. Based on the list of differential diagnosis (i.e., plausible diagnostic possibilities), neuroimaging, usually a brain MRI, and various investigations including genetic testing are usually performed as part of the evaluation of these patients to reach a specific diagnosis. General physical rehabilitation therapy and disease-specific treatments are available. Novel drugs and noninvasive cerebellar stimulation techniques are showing an early therapeutic promise for the symptomatic treatment of some of the cerebellar symptoms.

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Epidemiology of Cerebellar Disorders

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Abstract This chapter explains briefly the epidemiology of several cerebellar disorders, many of which are considered rare, and various risk factors associated with their development. For many cerebellar disorders, prevalence and incidence rates are unknown, or the values have been underestimated; this is true both at the global and regional levels. Scant epidemiological information can be attributed to lack of healthcare systems in various parts of the world, inaccurate classification of disorders in published studies, broad inclusion criteria, or simply rarity of the particular disorder. Information about the prevalence, incidence, or number of cases is important for planning and provision of services to address the needs of affected individuals. Epidemiological studies are also necessary to identify factors that contribute to the development of the disorder, which can be used to prevent or reduce the risk of developing the conditions at the population level.

Keywords Cerebellar disorders • Epidemiology • Incidence • Prevalence • Risk factors

Introduction

This chapter is focused on epidemiology of cerebellar disorders, and provides information on global and local prevalence and incidence (where available), and risk factors associated with the development of the disorder. *Prevalence* and *incidence* are the two main measures of disease occurrence in populations. Prevalence refers to the proportion of population with the condition of interest at a certain point in time or within a specific period. Incidence refers to the rate at which new events or cases of the condition of interest occur in a population in a defined period. Prevalence estimates provide useful information for planning and provision of services to address the needs of persons living with the conditions of interest. This information

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can also be used to examine trends in the occurrence of the conditions of interest to determine if the number of cases and rates have increased, decreased, or remained stable over time. Results of incidence studies are of great use for predicting future needs and for investigating causality and identifying factors associated with increased risk of a disorder of interest. Information on factors found to be significantly associated with the risk of the cerebellar disorders could aid in the identification of modifiable risk factors to prevent or reduce the risk of developing the condition at the population level.

Selection of Studies

A search was performed on the MEDLINE, Embase, and Scopus databases. The following search terms were used: [name of condition], AND "epidem*" OR "prevalence" OR "incidence" OR statistic* OR risk*. For the name of the condition, truncation was used to be more inclusive of alternative terms and spellings (e.g., cerebell* was used as appropriate for the cerebellum OR cerebellar). The search was restricted to records published in English. In total, 366 references were located. Initial examination of the records revealed that only 29 references to book, chapters, and sections and 337 references to articles were relevant to epidemiology or risk factors.

We selected studies providing estimates of prevalence and/or incidence for a specified population in a defined geographical region. Because we expected few publications for many of the conditions of interest, we defined broad inclusion criteria: (1) the article must mention estimates of prevalence and/or incidence and/or describe cases of the condition, or (2) the article must identify and describe risk factors for the condition, and (3) only articles published in English. Two authors independently reviewed the titles and abstracts of the publications identified by the initial search strategy. Studies that clearly did not meet the inclusion criteria were excluded, and the remaining studies were examined further. Inclusion was based on agreement between two reviewers; in cases of non-consensus, a third (and sometimes fourth) review was obtained for decision. For selected articles, data were extracted using a predefined data extraction form, which included the following parameters: publication type, geographical area, study population, number of patients identified, research design, study period, data source, condition and subtypes, prevalence and incidence estimates for each condition, and risk factors. Study limitations were noted. For conditions in which prevalence and incidence estimates were not available, the number of cases of a condition was reported. The reference lists of the selected papers were examined for additional studies. The quality of the studies was not assessed.

Results

Many cerebellar disorders are described as rare, very rare, or extremely rare. According to the consortium of European partners [1], "rare" is defined as affecting 1 per 2,000 people. Similarly, the United States Rare Diseases Act of 2002 [2]

defines "rare" as "any disease or condition that affects fewer than 200,000 people in the United States" or about 1 per 1,500 people. In Japan, a "rare" disorder is one that affects fewer than 50,000 people or 1 per 2,500 people.

Autism Spectrum Disorder and the Cerebellum

Autism spectrum disorders (ASD) are neurodevelopmental conditions that are characterized by deficits in social communication and social interaction, restricted and repetitive patterns of behavior, interests, or activities [3]. The comorbidity of ASD and intellectual disability (ID) is relatively low, with approximately 31% of US children with ASD being identified as having intellectual disability (i.e., IQ≤70 [4]). The cerebellum is reported to be one of the key brain regions affected in autism [5].

ASD are responsible for 0.3% of the global burden of disease and more than 7.6 million disability-adjusted life years. The global prevalence of ASD is estimated to be one person in 160 [6]. A large number of epidemiological studies from developed countries have investigated ASD prevalence, and less is known about prevalence of ASD in developing countries. Variable estimates of ASD prevalence are reported, ranging from 0.4 to 22.4 per 1,000, depending on the age, sex, and race/ethnic composition of the population studied, ASD diagnostic criteria used, changes in diagnostic criteria over time, the methods of data collection, and case ascertainment. While earlier European studies reported ASD prevalence estimates of 1 in 2,500 children across all ages in the population [7], more recent estimates of ASD prevalence based on large survey data suggest that 1–2% of all children are affected [8, 9]. For example, a UK school-based survey reported 99 per 10,000 [10]. The most recent estimate of ASD prevalence for children aged 3–17 years in the United States was reported at 2.24% based on data from the 2014 National Health Interview Survey (NHIS) [11]. The estimated prevalence was significantly higher than the estimated prevalence of 1.25% based on earlier years of data from the same survey (2011–2013). The observed difference was attributed to the change in wording of the survey questions that allowed parents to better differentiate ASD from other types of developmental disabilities [11]. Other studies from Europe, North America, and Asia also reported prevalence estimates of higher than 2% [4, 12, 13].

The Autism and Developmental Disabilities Monitoring (ADDM) Network is an active surveillance system in the United States, which provides estimates of the ASD prevalence among children aged 8 years living in 11 ADDM sites. According to this source, the overall prevalence of 8-year-olds with ASD in 2010 was 14.7 per 1,000 (1 in 68) [4]. There was variation in the reported prevalence estimates by sex and racial/ethnic background. ASD was four to five times more prevalent in boys (1 in 42 boys) than in girls (1 in 189 girls). Non-Hispanic white children were also 30% more likely than non-Hispanic black children to be identified with ASD. The median age at first ASD diagnosis was 53 months and did not differ by sex or race/ethnicity [4].

The reported estimates of ASD prevalence in Canada are lower due to the different case ascertainment method used. The National Epidemiologic Database for the Study of Autism in Canada (NEDSAC) has been monitoring the prevalence of ASD in three Canadian provinces including Newfoundland and Labrador, Prince Edward Island, and Southeastern Ontario since 2003. Based on information from this database, the prevalence of ASD was estimated at 1 per 94 children. Based on data collected through 2008 in Newfoundland and Labrador and 2010 in Prince Edward Island and Southeastern Ontario, the estimated average annual percent increases in prevalence among children 2–14 years of age ranged from 9.7 (95% CI: 7.8–11.6) to 14.6% (95% CI: 11.3–18.0) [14]. See Table 1 for a summary of prevalence estimates for ASD.

Epidemiological data over the past few decades suggest an increase in ASD prevalence globally. Several explanations are provided for this apparent increase in ASD prevalence including changes in diagnostic criteria and broadening of the diagnostic spectrum, greater awareness about ASD conditions among parents and clinicians, better diagnostic tools that might have led to a higher rate of diagnosis, and better reporting of cases and surveillance systems. The observed increase in ASD prevalence could also be the result of true increase in incidence. Given the complexity of the issue, however, no conclusions regarding causes of increased prevalence of ASD can be made at this time.

Research suggests that a complex and variable combination of genetic and environmental factors influences early brain development, leading to ASD [15, 16]. For example, a higher concordance between monozygotic and dizygotic twins is consistently shown, suggesting a genetic link for ASD [17]. Other researchers have estimated that approximately10–15% of persons with autism have a specific genetic mutation (see [18]).

Recent epidemiological studies have shown a positive association between increasing parental age at conception and ASD risk in offspring (see [19] for a review); however, a review of US data concluded that parental age is a very small contributor to the observed increases in the prevalence of ASD [20]. Maternal illness and infection during pregnancy, extreme prematurity, very low birth weight, and complications during birth, particularly those involving periods of oxygen deprivation to the baby's brain, are reported as important risk factors for ASD [19, 21]. Mothers exposed to high levels of pesticides and air pollution may also be at higher risk of having a child with ASD (e.g., [22]), although the evidence for this assertion has been described as limited and of moderate strength (see [23]). Interestingly, maternal smoking is also not associated with increased ASD [24]. A significant positive association has been observed between ASD prevalence and socioeconomic status (SES), suggesting increased risk of ASD with increasing SES. This observed association likely reflects diagnostic biases and/or disparities that exist in accessing services for ASD assessment [25]. Findings from a small number of studies suggest that autism risk is reduced among children whose mothers ingested prenatal vitamins and folic acid, fish and fish oil supplements, and/or fatty acids in the months before and after conception (see [26] for a review). The information available on risk factors associated with ASD clearly suggests that there is no single cause of autism.

Table 1 Prevalence of autism spectrum disorders

	Study details				Population			
Author, date	Publication type/ research design	Country (region)	Study period	Data source	Age	Sex	*N*	Prevalence
World Health Organization, 2013	Report	Global	–	–	–	–	–	6.25/1,000
Baron-Cohen et al., 2009	Cross-sectional	UK	–	Special Educational Needs (SEN) register and survey	5–9 years	M and F	11,700	9.9/1,000
Zablotsky et al., 2015	Cross-sectional	US	2011–2014	Population-based	3–17 years	M and F	43,283	22.4/1,000
Autism and Developmental Disabilities Monitoring Network Surveillance Year 2010 Principal Investigators, 2014	–	US	2010	ADDM Network	8 years	M and F	–	14.7/1,000 (overall) 1/42 (boys) 1/189 (girls)
Ouellette-Kuntz et al., 2014	Retrospective	Three Canadian provinces	2008–2010	The National Epidemiologic Database for the Study of Autism in Canada (NEDSAC)	2–14 years	M and F	–	10.6/1,000

ADDM Autism and Developmental Disabilities Monitoring

Ataxia

The word ataxia is derived from the Greek word "a taxis" meaning "without order." Patients with ataxia suffer from lack or loss of movement coordination resulting in poor coordination of gait or hands and disturbances in speech and oculomotor control [27]. Ataxia can negatively influence a person's ability to walk, sit, and stand [28]. The prevalence of ataxia in children has been estimated to be 26 per 100,000 [27], and the lifetime prevalence rate is reported to be 50 per 100,000 (see [29]), but these estimates vary depending on the type of ataxia or region. The most common types of ataxia are cerebellar, sensory, and vestibular. Cerebellar ataxia can also be divided into hereditary and nonhereditary ataxias.

Hereditary Ataxias

Hereditary cerebellar ataxias (HCA) can be inherited in an autosomal recessive, autosomal dominant, X-linked, and mitochondrial manner.

Autosomal Recessive Ataxias

In their systematic review and meta-analysis of prevalence in 22 studies, reporting on 14,539 patients from 16 countries, published between 1983 and 2013, Ruano, Melo, Silva, and Coutinho [30] reported that the prevalence rates for autosomal recessive hereditary cerebellar ataxia (AR-HCA) ranged from 0.0 to 7.2 per 100,000. Studies from this review are briefly described here. Two hospital-based studies from Cantabria region in Spain and Alsace region in France reported the highest prevalence rates at 7.2 per 100,000 [31] and 5.3 per 100,000 [32]. Prevalence estimates from multisource studies (i.e., cases from community settings, hospitals, and probands' families included in the estimates) tended to be lower (e.g., 2.3–4.8 per 100,000). For example, in a cross-sectional study conducted in southeast Norway between January 2002 and February 2008, Erichsen, Koht, Stray-Pedersen, Abdelnoor, and Tallaksen [33] found that the prevalence of AR-HCA was 2.3 per 100,000; patients were on average 32 years (range, 4–71 years) and were diagnosed at 9 years of age (range, 1–55 years). Gender differences in prevalence have not been observed. Globally, the incidence rate for AR-HCA is 4 per 100,000 (see [34]). See Table 2 for a summary of statistics found in studies examining the epidemiology of ataxia.

AR-HCA can be grouped into four classes based on the age of onset and key phenotypic features: Friedreich ataxia and early-, adolescent-, and adult-onset ataxias [34]. Friedreich ataxia is the most common form of AR-HCA in the world ([30]; see [35]). Some reports indicate that nearly 50% of all AR-HCA cases comprise Friedreich ataxia; therefore, screening all patients suspected of having AR-HCA for Friedreich ataxia prior to other genetic testing has been recommended [34]. In a retrospective cross-sectional study conducted in Iran, Friedreich ataxia and spinocerebellar ataxia (a type of adolescent-onset ataxia) were the most common types of

Table 2 Prevalence, incidence, and/or number of cases reported in studies examining the epidemiology of ataxia

	Study details				Population					
Author, date	Publication type/research design	Country (region)	Study period	Data source	Age	Sex	*N*	Prevalence	Incidence	Number or % of cases
Anheim et al., 2010	Retrospective	France (Alsace)	2002–2008	Hospitals	–	M and F	95	AR = 5.3/100,000	–	–
Erichsen et al., 2009	Cross-sectional	Norway (Southeast)	2002–2008	Population-based	0–80 years	M and F	171	AR = 2.3/100,000 AD = 4.2/100,000	–	–
Farghaly et al., 2011	Community-based	Egypt	–	Door-to-door survey	4–72 years	M and F	62,583	Acquired ataxia = 27.16/100,000	–	17 cases (7F; 10 M)
Polo et al., 1991	Retrospective	Spain (Northern)	1974–1986	Hospitals, families	M and F	54	AR = 7.2/100.000	–	–	
Nafissi et al., 2009	Retrospective cross-sectional	Iran	1993–1999	Dr. Shariati Hospital, University of Tehran	6–73 years	M and F	135	–	–	15 cases of HCA
Salman et al., 2013	Retrospective	Canada (Manitoba)	1991–2008	Children's hospital	0–16 years	M = F	184	Chronic ataxia = 2.4/10,000	Chronic ataxia = 3.2/100,000	9 cases of mitochondrial disease

AD autosomal dominant, *AR* autosomal recessive, *HCA* hereditary chronic ataxia

HCA identified among 135 patients with cerebellar ataxia from March 1993 to March 1999 in Dr. Shariati Hospital, University of Tehran [36]; however, the percentage of cases of the 15 patients with HCA was not reported. Other reports have estimated lower prevalence of Friedreich ataxia at 0.15 per 100,000, but higher rates for early-onset ataxias (i.e., 0.4 per 100.000 for ataxia telangiectasia) [33].

Autosomal Dominant Ataxias

In their review, Ruana and colleagues [30] found significant variation in the reported prevalence estimates for autosomal dominant HCA (AD-HCA) across 15 studies. Overall, prevalence of AD-HCA was 2.7 per 100,000 (range, 0–5.6 per 100,000). No cases of AD-HCA were found among 16 Italian patients with hereditary ataxia [37], whereas other work conducted in Portugal suggests a prevalence rate of 5.6 per 100,000 population [38]. Prevalence rates in multisource population-based surveys (e.g., [38]) or in the registry studies (e.g., [39]) were higher than genetic center-based studies ranging from 1.6 to 2.5 per 100,000. In the Netherlands, the prevalence of the AD-HCA is at least 3 per 100,000 [40]. In a cross-sectional study conducted in southeast Norway between January 2002 and February 2008, the prevalence rate of AD-HCA was estimated at 4.2 per 100,000, and only 8% of cases had a genetic diagnosis [33]. The mean age of the cases of AD-HCA was 57 years (13–94 years) without any gender difference after adjustment for age [33]. Among patients with AD-HCA, spinocerebellar ataxia type 3 (SCA3)/Machado-Joseph disease may be the most common, followed by SCA2 and SCA6 (see [30]). See Table 2.

X-Linked Ataxias and Ataxias Due to Mitochondrial Mutations

Little information is available on the epidemiology of X-linked and mitochondrial HCA. This may be due to the fact that the required genetic testing for the diagnosis of these conditions is not performed. The few studies that describe X-linked ataxias or ataxias linked to mitochondrial mutations have only found few cases. For example, in a retrospective study using multiple sources of data of children examined at a children's hospital in Manitoba, Canada, Salman, Lee, Tjahjadi, and Chodirker [41] reported nine cases of intermittent or chronic ataxias in children linked to mitochondrial disorder. Therefore, further epidemiological studies are required to determine the extent to which X-linked and mitochondrial HCA occur. See Table 2.

Acquired Ataxias

Acquired ataxias are a group of nonhereditary ataxias associated with exposure to alcohol or other toxins or infections or can be due to vitamin deficiency or metabolic disorders [42]. Acquired ataxias are typically divided into two main groups: acute (in a period of minutes to hours it occurs) and subacute (onset is from days to weeks).

Our literature search revealed only one original research study describing the epidemiology of acquired ataxia. In a population-based study, Farghaly and colleagues [29] estimated the crude prevalence rate of acquired ataxia to be 27.16 per 100,000 in Al-Kharga district, New Valley, Egypt. Using a door-to-door survey method, 17 cases of acquired ataxia were identified; on average, individuals were 31.8 years of age (range, 4–72 years) and the male to female ratio was 2.1:1 [29]. See Table 2.

Describing the prevalence rates of acquired ataxia by age group is important because risk factors for the condition often differ across age. In a retrospective study conducted at a children's hospital in Pittsburgh, USA, Thakkar and colleagues [43] reported that post-infectious cerebellar ataxia was a common cause of acute cerebellar ataxia (ACA) affecting 59% of patients with ACA. The authors reported no cases of ACA related to varicella infections; however, other research has indicated that varicella and other infections are strongly associated with ACA [44]. Post-infectious cerebellar ataxia accounts for up to 40% of ACA in preschool children (age 1–4 years), which is followed by toxic ingestion (i.e., 30% of ACA cases) [28]. Strokes (ischemic or hemorrhagic) and medications are other potential causes of ACA, particularly in elderly individuals [29]. Subacute ACA can be observed in various situations, including nutritional deficiencies (vitamin B12, vitamin E, folate, copper), autoimmune or inflammatory diseases, and infectious, primary, and metastatic tumors [34].

Cerebellar Tumors

Primary brain tumors are the most common type of neoplasms of childhood, comprising approximately 20% of all pediatric tumors. Globally, about 30,000–40,000 children develop central nervous system tumors each year (see [45]). In the United States, over 3,000 children under the age of 20 years are diagnosed with a brain or spinal cord tumor annually [46]. The incidence of brain tumors in children is estimated between 2.76 and 4.28 per 100,000 children per year [47]. Although significant progress has been made in the diagnosis and treatment of brain tumors in children, they are still the primary cause of cancer-related deaths in children. Tumor type and location are important prognostic factors. Tumors of the cerebellum are associated with symptoms such as ataxia, horizontal nystagmus, dysmetria, headache, vomiting, and lethargy [47, 48].

Medulloblastoma

Medulloblastomas, which typically arise in the cerebellum, are the most common malignant central nervous system tumor in children and the second most common pediatric brain neoplasm. Medulloblastoma accounts for 12–25% of all central nervous system tumors in children [46]. Medulloblastomas present at approximately

5–7 years of age on average and occur more frequently in boys than in girls [49–51]. Moreover, 25% of newly diagnosed cases of medulloblastoma occur in individuals aged 19 years and older [52]. The earlier incidence estimates of medulloblastoma brain tumor were 9.6 per million in children and 0.54 per million in adults [53, 54]. The European annual incidence rate for primitive neuroectodermal tumors (PNET, morphologically similar tumors arising in other areas of the central nervous system) was reported to be 6.5 per million children (age 0–14 years) for the period 1988–1997 [55]. The incidence rates of medulloblastoma and PNET are fairly stable from birth through 3 years of age and decline gradually thereafter. See Table 3 for a summary of statistics.

Several studies from Asia have examined the epidemiology of cerebellar tumors. In a retrospective cohort study, Tabatabaei and colleagues [47] reviewed the medical records for all pediatric cases of posterior fossa tumor that were referred to a neurosurgical clinic in Iran for surgery from 1981 to 2011. The authors extracted demographic data including patient's age, gender, and tumor characteristics along with the location and pathological diagnosis for all the cases and assessed the surgical outcomes according to pathological diagnosis. The study cohort consisted of 84 patients (52 males, 32 females). Medulloblastoma was found in 42.8% of cases, followed by cerebellar astrocytoma (28.6%), ependymoma (14.3%), brain stem glioma (7.2%), and miscellaneous pathologies (e.g., dermoid, and tuberculoma) (7.2%).

Ahmed and colleagues [56] examined the epidemiology of brain tumors during infancy and childhood using 10 years of data (1989–1998) at a tertiary care hospital in Karachi, Pakistan. Of the 81 cases that were identified, 71.6% were males and 28.4% were females (i.e., male to female ratio of 2.5:1). When dividing the cases into three age groups (0–4, 5–9, 10–14 years), the largest number of cases was found in children ages 5–9 years. The mean age for all cases was 8.8 years (95% CI: 7.9; 9.6) with a marginal variation for cases occurring in the cerebrum and cerebellum. Of the 81 cases, 33.3% were supratentorial, and 66.7% were infratentorial tumors, and 70.4% of the infratentorial tumors were medulloblastomas. Consistent with other research [50], Ahmed and colleagues [56] concluded that pediatric brain tumors are more prevalent among males than females and that medulloblastoma is the most common type of brain tumors in children. Similarly, Asirvatham and colleagues [49] found that medulloblastomas were the second most common type of brain cancers (11.4% of cases) among 1,403 tumors that were identified in children (aged 0–18 years) diagnosed between 1990 and 2004 at a tertiary care center in South India. The mean age at diagnosis was 10.9 years, and males were more frequently diagnosed than females (i.e., ratio of 1.7:1).

Chan and colleagues [57] conducted a 9-year retrospective study based on data reported to the Singapore Children's Cancer Registry from 1997 to 2005. A total of 39 children aged 15 years and younger were diagnosed with medulloblastoma or PNET arising in the cerebellum. Follow-up data for these children were collected up to 2006. Medulloblastoma/PNET was the most common type of brain tumor in the sample, accounting for 40.7% of all brain tumors.

Table 3 Prevalence, incidence, and/or number of cases reported in studies examining the epidemiology of cerebellar tumors

	Study details				Population					
Author, date	Publication type/research design	Country (region)	Study period	Data source	Age	Sex	*N*	Prevalence	Incidence	Number or % of cases
Ahmed et al., 2007	Cross-sectional	Pakistan	1989–1998	Hospital database	M_{age} = 8.8 years; range: 5–9 years	M = 71.6%; F = 28.4% M/F = 2.5/1	81	–	–	–
Asirvatham et al., 2011	Retrospective cohort	India	1990–2004	Pathology and medical records	M_{age} = 10.9 years; range: 0–18 years	M/F = 1.7/1	1043	–	–	5 most frequent tumors: astrocytoma (47.3%), medulloblastoma (11.4%), craniopharyngioma (9.7%), ependymal tumors (4.8%), nerve sheath tumors (4.1%)
Chan et al., 2007	Retrospective cohort	Singapore	1997–2005	Singapore Children's Cancer Registry	0–15 years	M and F	39	–	–	Medulloblastoma/PNET = 40.7% of tumors
Giordana et al., 1999	Retrospective cohort study	Italy	1976–1995	Hospital charts and operating room books from 5 neurosurgical units	16–69 years	M and F	4.3 million	–	All ages: 0.5/million/year; males (0.82/million/year); females (0.28/million/year); highest incidence for 16–19 years old (2.33/million/year)	45 (32M; 13F) cases of medulloblastoma

(continued)

Table 3 (continued)

	Study details				Population					
Author, date	Publication type/research design	Country (region)	Study period	Data source	Age	Sex	*N*	Prevalence	Incidence	Number or % of cases
Peris-Bonet et al., 2006		Europe	1978–1997	ACCIS database	0–14 years	M and F	19,531		ASR for CNS tumors (1988–1997) = 29.9 per million; ASR for PNET = 6.5 per million (1988–1997)	–
Roldán et al., 2008	Retrospective cohort study	Canada	1975–1996	Alberta Cancer Registry	Age of diagnosis: children M_{age} = 7 years; adults M_{age} = 29.2 years	M>F	2.8 million		–	49 cases of medulloblastoma/PNET
Smoll and Drummond, 2012	Retrospective cohort study	USA	1973–2007	Epidemiology and End Results (SEER) database	Children 1–9 years 10 times more affected by medulloblastoma and 4.6 times more by PNET	M/F = 1.58/1		–	Medulloblastoma = 1.5 per million population, PNET = 0.62 per million population	1,372 cases of medulloblastoma, 530 cases of PNET
Tabatabaei et al., 2012	Retrospective cohort study	Iran	1981–2011	Patient records	1–14 years	M>F	84	–	Brain tumors = 2.76–4.28/100,000 children per year	Medulloblastoma in 42.8% of cases
Thorne et al., 1994	Retrospective population-based study	England (Southwest and Northern)	1976–1991	Bristol Registry of Childhood Cancer	0–14 years	M and F	20.0 million child yrs		1976–1984 = 9.6 per million per year; 1985–1991 = 1.7 per million per year	1976–1984 = 16 cases of medulloblastoma 1985–1991 = 2 cases of medulloblastoma

ASR age-standardized incidence rate, *CNS* central nervous system, *PNET* primitive neuroectodermal tumor

Several studies from North America provided estimates of prevalence and/or incidence rates of cerebellar tumors. Using data from the Surveillance, Epidemiology, and End Results (SEER) database, Smoll and Drummond [50] estimated the incidence rates, ratios, and time trends of medulloblastoma and PNET in children and adults in the United States. Between 1973 and 2007, 1,372 people were diagnosed with a medulloblastoma and 530 with a PNET. The overall incidence rate of medulloblastoma and PNET was estimated at 1.5 and 0.62 per million, respectively; children (1–9 years of age) were ten times more likely to be diagnosed with these tumors than adults (i.e., 6.0 vs. 0.6, respectively). Children were also 4.6 times more likely to be afflicted by a PNET than adults. During childhood, boys were 1.58 times more likely than girls to be diagnosed with a medulloblastoma. Those categorized as "black" were 0.61 times more likely than those classified as "white" to be diagnosed with a medulloblastoma, and this was significant in children and adults [50]. Roldan and colleagues [58] examined 21 years of data (1975–1996) from the Alberta Cancer Registry, a population-based cancer registry for the province of Alberta in Canada, which had a population of 2.8 million in 1996. An addition has been made to clarify that 61% of the total sample of 49 cases were males. The mean age at the diagnosis for children was 7 years and for adults was 29.2 years.

Although some genetic disorders (i.e., Gorlin syndrome, Turcot syndrome, Li-Fraumeni syndrome [LFS]) are associated with an increased risk of medulloblastoma, the etiology is unknown for most patients [59]. Because the highest incidence rate is reported during childhood, very early life experiences may be contributing factors in the development of brain tumors [51]. A meta-analysis conducted by Harder and colleagues [60] confirmed that high birth weight was associated with increased risk for medulloblastoma. Infection during pregnancy and deficient social environment may also be significant risk factors for cerebellar tumors. For example, in a case-control study in England, children of mothers with a documented viral infection during pregnancy had an 11-fold increased risk of malignant nervous system tumor compared to children whose mothers did not have such a history during their pregnancy [61]. Lack of social contact in the first year of life is associated with increased risk of developing a central nervous system tumor in childhood, and the effect is greater for medulloblastoma/PNET [62].

The role of diet as a potential risk or protective factor in brain tumors has been investigated in several studies [e.g., 63, 64, and 65]. In rodents, maternal dietary intake of N-nitroso compounds (NOC) and NOC precursors (e.g., sodium nitrite, amines, and amides) during pregnancy is believed to increase the risk of brain tumor in offspring (e.g., [66]). A large international collaborative case-control study on childhood brain tumors reported that foods associated with increased risk of brain tumors were cured meats, eggs/dairy, and oil products; however, yellow-orange vegetables, fresh fish, and grains reduced the risk significantly [64].

Studies based on a very small sample sizes have also reported that exposure to electromagnetic fields is a potential risk factor for childhood brain tumor [67]. However, in the large-scale United Kingdom (UK) Childhood Cancer Study, the authors found that exposure to electromagnetic fields was not linked to childhood brain tumors [68]. A recent large Canadian study examined the contribution of

maternal occupational exposure to extremely low-frequency magnetic fields shortly before and during pregnancy on the incidence of childhood brain tumors. A significantly increased risk was observed for astroglial tumors as well as for all childhood brain tumors, but no association was specifically assessed for medulloblastoma/PNET [69].

Several epidemiological investigations have examined the association between parental exposure to pesticide and childhood brain tumors, with the majority reporting positive associations. For example, in a recent population-based case-control study, the association between brain cancer in children and parental exposure to pesticides in occupational and residential settings was investigated [70]. The researchers reported very weak association between PNET for any of the pesticide classes or exposure sources considered. However, Rosso and colleagues [71] found an association between household exposure to chemicals and medulloblastoma/PNET in children registered with Children's Cancer Group (USA and Canada), particularly for pesticides used in lawn care. A European study found an increased risk of PNET with parental exposure to polycyclic aromatic hydrocarbons (OR = 2.0, 95% CI: 1.0, 4.0) and high maternal exposure to solvent (OR = 3.2, 95% CI: 1.0, 10.3) during the 5-year period before birth [72].

Fetal Alcohol Spectrum Disorders (FASD)

Fetal alcohol spectrum disorders (FASD) are a group of conditions that occurs when alcohol was consumed during pregnancy. FASD are divided into several subgroups: fetal alcohol syndrome (FAS), partial fetal alcohol syndrome (pFAS), alcohol-related neurodevelopmental disorder (ARND), and alcohol-related birth defects (ARBD) [73]. Alcohol has irreversible effects on the central nervous system, including abnormal functioning of the amygdala, thinning of the corpus callosum, and reduced brain volume with specific reductions in the frontal lobe, striatum and caudate nucleus, thalamus, and cerebellum [73]. Growth deficiency (height and weight), central nervous system and neurological damage (memory problems, hearing loss, poor gait), and facial dysmorphism (a smooth philtrum, small palpebral fissures, and thin vermilion) are common features of patients with FASD [74].

A recent systematic review of FASD prevalence published in 2013 found significant variations in the reported prevalence estimates across the reviewed studies [75]. Ospina and Dennett [75] classified the 54 studies into six categories based on FASD (or subtypes) prevalence for a specific population. The FASD prevalence estimates for communities based on population-level data range from 0.2 to 5 per 1,000 population. Studies of FASD prevalence in school settings also reported variable estimates, ranging between 0.5 and 10.7%. The reported estimates of FASD prevalence among children in care were found to be much higher than the estimates for school settings or communities, ranging between 30.5 and 52%. A limited number of studies from North America examined FASD prevalence in correctional systems, providing estimates between 9.8 and 23.3%. The majority of studies that examined estimates of FASD prevalence in aboriginal populations were conducted in Canada.

The pooled estimate of FAS prevalence in aboriginal people based on six studies was 0.2% or 2 FAS cases per 1,000 population. The FASD prevalence in other specialized settings, for example, in special education settings, was found to be much higher. The pooled prevalence estimate of FAS was 4.9% (95% CI: 2.5, 7.3), and the pFAS prevalence was 5.4%. The great variation observed in the reported estimates could be in part due to the differences in the characteristics of the populations studied (e.g., age, sex, race/ethnicity, aboriginal status), diagnostic criteria used, methods of case ascertainment, and years of data used.

Popova and colleagues [76] conducted a systematic review and meta-analysis of FASD comorbidity in 2016. The authors identified 428 comorbid conditions in persons with FASD. The identified comorbid conditions extended over 18 of 22 chapters of the ICD-10. The comorbid conditions with the highest prevalence were those related to peripheral nervous system and special senses, conduct disorder, receptive language disorder, chronic serous otitis media, and expressive language disorder.

Cerebellar Malformations

Cerebellar Agenesis

Cerebellar agenesis is an extremely rare condition with complete absence of the cerebellum or with only a small portion of the cerebellum (subtotal cerebellar agenesis) [77, 78]. Primary cerebellar agenesis has a high mortality rate and is typically identified during autopsy. Cerebellar agenesis negatively affects motor skill development, but may improve with age, and has been associated with abnormalities of non-motor functions, such as expressive language, affective behavior, neurological abnormalities, and working memory [79].

Our initial search of studies providing estimates of prevalence and/or incidence, or number of cases, resulted in only one article describing one new case of complete primary cerebellar agenesis [79]. Yu and colleagues [79] described the clinical presentation and subsequent imaging tests of a 24-year-old female, who was married with a daughter, and was living in China. Review of the article revealed seven other publications describing eight living cases of cerebellar agenesis, ranging in age from 4 months to 59 years [77, 80–85]. Interestingly, some individuals with total or subtotal cerebellar agenesis were described as being asymptomatic and having typical neurobehavioral, mental, and physical functioning [81]. See Table 4 for a summary of study statistics.

Dandy-Walker Malformation

Dandy-Walker malformation (DWM) is a complex developmental anomaly involving the fourth ventricle and cerebellum, characterized by an enlargement of the fourth ventricle, vermian agenesis (partial or complete), and posterior fossa cysts

Table 4 Prevalence, incidence, and/or number of cases reported in studies examining the epidemiology of cerebellar malformations

	Study details				Population					
Disorder Author, date	Publication type, research design	Country (region)	Study period	Data source	Age	Sex	*N*	Prevalence	Incidence	Number of cases or families
Cerebellar agenesis										
Sener and Jinkins, 1993	Case report	Texas, USA	–	University of Texas Health Science Center	58 years	F	–	–	–	1 case
Sener, 1995	Case report	–	–	–	6 years	1F;1M	–	–	–	2 cases
Tekin et al., 2002	Case report				7 years	F	–	–	–	1 case
Timmann et al., 2003	Case report	Germany	–	–	59 years	F	–			1 case
Van Hoof and Wilmink, 1996	Case report				46 years	M	–	–	–	1 case
Velioglu et al., 1998	Case report	–	–	–	22 years	M	–	–	–	1 case
Yu et al., 2014	Review, Case report	China	–	General Hospital of Jinan Military Command	24 years	F	–	–	–	1 case
Dandy-Walker malformation										
Di Bella and Pizzo, 2010	Original research, retrospective	Italy (Catania)	1999–2009	–	Pediatric	–	5,000	32/10,000	–	16 cases
Hakami and Majeed-Saidan, 2011	Retrospective analysis of prospectively collected data	Saudi Arabia	2001–2010	Neonatal Intensive Care Unit in Riyadh Military Hospital, Riyadh	–	–	94,210	2.3/10,000	–	–
Kontopoulos et al., 2008	Retrospective	Florida, USA	1997–2005	–	–		600 monochorionic twins	–	1/8,000–10,0000 live births	10 cases

McClelland et al., 2015		USA (22–36 states)	1997–2003	Kids' Inpatient Database	–		–		1.36/1,000	–
Ohaegbulam and Afifi, 2001	Retrospective analysis of prospectively collected data	Saudi Arabia (Northern)	1989–1999	Population of military personnel and their dependants	–		45,274 live births	–	1/10,000 live births per year; males (1.24/10,000), females(0.78/10,000)	–
Joubert syndrome										
Akhondian et al., 2013	Case report	Iran	–	–	12, 10, 3 years	2F; 1M	3	–	–	3 cases in one family
Brancati et al., 2010	Review	–	–	–	–	–	–	–	1:18,000–1:100,00	
Hakami and Majeed-Saidan, 2011	Retrospective analysis of prospectively collected data	Saudi Arabia	2001–2010	Neonatal Intensive Care Unit in Riyadh Military Hospital	–	–	94,210	1.7 per 10,000	–	–
Lissencephaly and cerebellar hypoplasia										
Koul et al., 2006	Case report	Oman	1993–2003	NR	15 days to 6 years	4F; 8M	40	–	–	12 cases; family history of developmental delay in 2/7 cases
Ozyurek and Kose, 2005	Case report	Turkey	–	–	3 years	M	1	–	–	1 case
Pontocerebellar hypoplasia										
Alkan et al., 2009	Retrospective study of MRI and CT images	Turkey	2002–2008	–	8.9 years (30 weeks gestation – 17 years)	22F; 23F	45	–	–	12 cases

(continued)

Table 4 (continued)

	Study details				Population					
Disorder Author, date	Publication type, research design	Country (region)	Study period	Data source	Age	Sex	*N*	Prevalence	Incidence	Number of cases or families
Grellner et al., 2000	Case report	Germany	–	–	1.5 years	M	1	–	–	1 case
Zafeiriou et al., 2013	Retrospective cases study	Greece	–	Tertiary hospital	24–28 weeks (mean, 25.8 weeks)	7F; 5MF	12	–	–	12 cases observed in extreme prematurity
Gómez-López-Hernández (GLH) syndrome										
Abdel-Salam et al., 2014	Case report	Egypt	–	–	24 months	M	1	–	–	1
De Mattos et al., 2014	Review, case report	Brazil	–	–	Minutes old	M	1	–	–	1
Erzin et al., 2016	Letter, case report	Turkey	–	–	24 years	M	1	–	–	1
Fernandez-Jaen et al., 2009	Review, case report	Spain	–	–	14 months; 6 years	M	2	–	–	2
Gomy et al., 2008	Review, case report	Brazil	–	–	12 years; 29 years	M	2	–	–	2
Kobayashi et al., 2015	Case report	Japan	–	–	42 weeks gestational age; then at 4 and 39 years	F	1	–	–	1
Poretti et al., 2008	Case report	Switzerland	–	–	38 weeks gestation to 39 years	2F;2 M	4	–	–	4 cases
Rush et al., 2013	Case report	USA	–	–	4–11 years	3F;1M	4	–	–	4 cases
Saricam et al., 2015	Case report	Turkey	–	–	16 years	F	1	–	–	1 case

Schell-Apacik et al., 2008	Case report	Germany	–	–	3.67 and 15.67 years	M	1	–	–	1 case
Sukhudyan et al., 2010	Case report	Armenia	–	–	1.5–20 years	M	6	–	–	6 cases
Rhombencephalosynapsis (RS)										
Arisoy et al., 2016	Case report	Turkey	–	–	19 weeks	–	–	–	–	1 fetus
Passi et al., 2015	Case report	India	–	–	4 years	M	1	–	–	1 case
Weaver et al., 2013	Retrospective, case report	USA	–	–	10 months–10 years	3F; 6 M	9	–	–	6 cases of RS; 3 cases of partial RS
Sener et al., 2000	Retrospective review of MRI examinations	Turkey	–	–	1 day–18 years	–	3,000	13 per 10,000	–	6 cases reported (3 months–8 years, 3F, 3 M)
Utsunomiya et al., 1998	case report	Japan	–	–	3 years, 4 years	2 M	–	–	–	2 cases
Chiari malformation										
Di Bella and Pizzo, 2010	Original research, retrospective	Italy (Catania)	1999–2009	–	Children	M/F	5,000	32 per 10,000	–	12 cases (0.24%)
Dughal et al., 2014	Prospective observational	Pakistan (Lahore)	2010–2012	–	Infants with anomalies	33F;47M	80	–	–	2 cases (3%)
Ghavami and Abedinzadeh, 2011	Original	Iran (East Azarbaijan)	2005–2008	–	–	–	22,500 pregnant women	–	–	41 fetuses
Lee et al., 2015	Retrospective review of surgical records	Korea	1991–2012		12–250 months	–	–	–	–	54 cases

(continued)

Table 4 (continued)

	Study details				Population					
Disorder Author, date	Publication type, research design	Country (region)	Study period	Data source	Age	Sex	*N*	Prevalence	Incidence	Number of cases or families
Meadows et al., 2000	Retrospective analysis of MR images	USA (Maryland)	1994–1997	Imaging report database, Johns Hopkins Hospital	0–70 years of age	–	22,591	7.8 per 10,000	–	0.77% CMI
Sakushima et al., 2012	Survey	Japan	2008–2009	Nationwide postal survey	38 ± 23.5 years	56.5%F; 42.1%M	708	–	–	CMI: 48.0%; CMII: 8.1%
Schanker et al., 2011	Case report	USA	–	–	20–62 years	5F;1M	–	–	–	CMI: 3 family pairs (2 mother-daughter; 2 father-daughter)
Tectocerebellar dysraphia										
Agrawal et al., 2010	Case report	India	–	Datta Meghe Institute of Medical Sciences	3 months	M	1	–	–	1
Anik et al., 2010	Case report	Turkey	–	Kocaeli University	5 months	F	1	–	–	1
Chowdhary et al., 1989	Original research, case report	Saudi Arabia	–	King Faisal University	1 week to 5 months	3F, 1M	4	–	–	4 cases
Friede, 1978	Case report	Switzerland	–	University of Zürich	2 months, 8 years, newborn	1F; 2M	3	–	–	3 cases
Krishnamurthy et al., 2008	Case report	India	–	Maulana Azad Medical College	7 months	M	1	–	–	1 case
Poretti et al., 2011	Case report	USA	–	The Johns Hopkins School of Medicine	4 years	F	1	–	–	1 case

CMI Chiari type I malformations, *CMII* Chiari type II malformations

[86–88]. Hydrocephalus is a common finding in DWM cases and can lead to death if not treated quickly [89].

A number of studies conducted in the United States [90, 91], Italy [92], and Saudi Arabia [93] have examined the epidemiology of DWM. All of the studies had retrospective designs, with sample sizes ranging between 129 and 14,599.

Di Bella and Pizzo [92] examined the health records of 5,000 children referred to a pediatric radiology unit at the University Hospital of Catania, Italy, for diagnostic procedures over a 10-year period (January 1999–October 2009). The authors found 16 cases of DWM, ranging in age from 1 month to 9 years (ten males, six females). Thus, based on this study, the prevalence of DWM was estimated at 32 per 10,000 population.

In a retrospective analysis of prospectively collected data on all newborns admitted to the neonatal intensive care unit in Riyadh Military Hospital, Riyadh, Saudi Arabia, Hakami and Majeed-saidan [93] reported that 22 infants were identified with Dandy-Walker anomaly (incidence, 2.3 per 10,000). This rate was higher than that estimated in a population of military personnel and their dependents in the Northern region of Saudi Arabia. Ohaegbulam and Afifi [94] identified all infants diagnosed with DWM during an 11-year period (1989–1999) from a cohort of 45,274 live births. The incidence of DWM was 1 per 10,000 live births per year and was higher for males (1.24 per 10,000) than for females (0.78 per 10,000).

In the United States, the incidence of DWM has been estimated at 1.36 per 1,000 in study examining data from the Kids' Inpatient Database containing information from hospitals in 22–36 states covering 1997–2003 [89]. Another US study reported that the incidence of DWS in complicated monochorionic twins was approximately 200 times higher than that expected for the general population [90]. DWS was also more likely to occur in the smaller twin and more likely to be restricted in growth. Other research has shown that DWM is associated with maternal non-Hispanic black ethnicity, a history of infertility treatment, preterm birth, low birthweight, and twin births [95], but these findings have been inconsistent [96]. See Table 4.

Joubert Syndrome and Related Disorders (JSRD)

Joubert syndrome and related disorders (JSRD), originally described in 1968 as Joubert syndrome, is primarily an autosomal recessive neurologic disorder characterized by absence or hypoplasia of the cerebellar vermis and a malformation in the brain stem resulting in hypotonia, developmental delay, neonatal respiratory dysregulation, abnormal eye movements, ataxia, polydactyly, and intellectual disability [97–99]. Nephronophthisis (NPHP) or cystic renal dysplasia is seen in approximately one-quarter of cases of Joubert syndrome [100]. An important malformation is the "molar tooth sign" (MTS) – a pathognomonic midbrain-hindbrain malformation [101, 102].

Globally, the incidence of Joubert syndrome has been estimated at 1 per 80,000–100,000 live births, although some researchers suggest that this range may underestimate the actual number of cases of the syndrome [101]. For example, Srour and colleagues [103, 104] suggest that within the French-Canadian population, there is a higher prevalence of Joubert syndrome, particularly in the Saint Lawrence region of the province of Quebec, Canada. Akhonidan and colleagues [105] identified and described the same presentations of Joubert syndrome in three family members in Iran. In a Saudi Arabian study, Hakami and Majeed-saidan [93] (see also Dandy-Walker Malformation) found 22 cases of Joubert syndrome (incidence, 1.7 per 10,000 live births). Thus, it appears that ethnicity can be a risk factor for the condition.

Lissencephaly and Cerebellar Hypoplasia (LCH)

Lissencephaly and cerebellar hypoplasia (LCH) is a rare autosomal recessive disorder in which the cerebellum, cerebrum, and brain stem are affected [106]. Generally, lissencephaly is caused by impairment in neuron migration, which is essential for development of the cerebellar cortex. Consequently, the cerebellar cortex becomes smooth (lack of folia and sulci) [107]. Seizures, hypotony or spasm, and psychomotor retardation are the symptoms of LCH and death typically occurs at an early age. Affected individuals have moderate to severe intellectual disabilities and delayed development. Prevalence of LCH is largely unknown [108]. Koul, Jain, and Chacko [109] examined data from all children in Oman (population 2.3 million) from January 1993 to December 1997 and identified 12 cases of lissencephaly. In another report, researchers in Turkey described a case of Joubert syndrome with lissencephaly [110] but the type of lissencephaly was not reported. Recently, Zika virus infection in pregnant mothers has been suggested as a risk factor for lissencephaly [111].

Pontocerebellar Hypoplasia (PCH)

Pontocerebellar hypoplasia (PCH) is a group of prenatal onset, autosomal recessive, neurodegenerative disorders that affects brain development [112]. Characteristic features of PCH include atrophy of the brain stem, particularly pons (pontine nuclei), and cerebellum, movement problems, intellectual disability, and communication difficulties (i.e., lack of ability to speak) [113]. Affected individuals die during infancy or childhood [114] before the age of 6 years [115].

The condition appears to affect males and females similarly and has been observed in infants born extremely prematurely [116]. About 100 cases of PCH have been reported in the literature [117]. Alkan, Kizilkilic, and Yildirim [118] conducted a retrospective study of the magnetic resonance imaging (MRI) scans and

computed tomography images from 45 children (22 girls, 23 boys; 30 weeks–17 years of age) of cerebellar malformation and found 12 cases with cerebellar hypoplasia. Grellner, Rohde, and Wiski [119] describe one case of PCH type 2 – a 1.5-year-old boy who had severe psychomotor delay and dyskinesia and epileptic seizures. The genetics of PCH are largely understood; as such, in a specific community in the Netherlands, genetic carrier screening has taken place to identify high-risk couples for having children with PCH [120]. Between September 2012 and 2013, Mathijssen and colleagues [120] identified 4 of 92 couples were carriers with a 1-in-4 risk of having a child with PCH type 2 in each pregnancy.

Gómez-López-Hernández Syndrome (GLH)

Gómez-López-Hernández (GLH) syndrome, also known as cerebellotrigeminal-dermal dysplasia, is a neurocutaneous disorder characterized by rhombencephalosynapsis (see Rhombencephalosynapsis section below) and trigeminal anesthesia [121]. GLH manifestations may include alopecia (partial or complete hair loss); hypotonia; wide-spaced eyes; ataxia; impaired pain sensation; low-set, posteriorly rotated ears; short stature; developmental delay; and seizures [122]. Although intellectual disability is typically observed, individuals with normal cognitive function have been described in the literature (e.g., [123]).

Only 34 cases of GLH have been reported worldwide. Cases have been described in Armenia [124], Brazil [125], Egypt [126], Germany [127], Japan [121], Spain [122], Switzerland [128], and Turkey [129, 130]. Because many of the cases described have occurred in Brazil, a "founder effect" has been suggested for GLH in Brazil [125]. Several researchers have argued that GLH may not be as rare as has been previously thought; they suggest that it is under-recognized in the pediatric population because clinical presentation varies in severity [121, 123, 128].

Suggested risk factors for GLH include smoking and cannabis use and the use of other drugs (i.e., valproate, ethosuximide) by mothers during pregnancy [131]. No specific mutation or chromosomal abnormality has been identified for GLH; however, the findings reported by de Mattos and colleagues [125], Gomy and colleagues [132], and Saricam and colleagues [130] suggest an autosomal recessive pattern of inheritance. Erzin and colleagues [129] report the only case of GLH with schizophrenia.

Rhombencephalosynapsis

Rhombencephalosynapsis is a rare midline brain malformation that involves cerebellar vermis absence, fusion (continuity) of the cerebellar hemispheres, and fusion of the dentate nuclei [133]. Rhombencephalosynapsis can occur in isolation or in combination with other anomalies such as Gómez-López-Hernández syndrome (see

above), VACTERL features, and holoprosencephaly [133–135]. Patients suffer from truncal ataxia, limb ataxia, head stereotypies, delayed motor development, abnormal eye movements, and other features determined by supratentorial abnormalities [136].

Some authors suggest that there are only about 30–35 cases of rhombencephalosynapsis that have been identified and described in the literature from 1914 to 1995 [135, 137], but there may be over 100 cases [138] reported in the literature worldwide. A number of studies from the United States [133, 139], Japan [135], India [138], and Turkey [137, 140] have been published. The majority of these studies are case reports describing one or two cases; however, Tully and colleagues [133] describe their comprehensive search for patients with RES in a database of more than 6,800 individuals with brain malformations and other developmental brain disorders. The authors found and described 53 cases of RES and the features of GLH, VACTERL, or other malformations that presented in conjunction with RES. Examination of the MRI scans of 3,000 children, Sener [137] estimated the prevalence of rhombencephalosynapsis to be 0.13% (13 per 10,000), a finding that was higher than expected. Clinicians generally recommend that differential diagnosis should be made from DWM and other anomalies [140].

Chiari Malformations

Chiari malformations are classified by type (types I–IV) based on the severity of the structural defects in the cerebellum, craniocervical junction, and brain stem [141]. In most cases, the posterior fossa is small resulting in downward displacement of the cerebellum and lower medulla together or the cerebellum alone into the spinal canal [142]. Consequently, cerebrospinal fluid can be blocked, and symptoms such as abnormal eye movements, headache, dizziness, muscle numbness, and problems with balance and coordination can be observed [143, 144]. Chiari type II malformations (CMII) are usually identified at or before birth [145] but may go undetected if symptoms are not apparent. This is often the case for Chiari type I malformations (CMI), which is frequency asymptomatic and may not be recognized until adolescence or adulthood [146]. The average age of a CMI diagnosis has been reported to be 24.9 ± 15.8 years [147].

Information on prevalence of Chiari malformations at the population level is lacking. Although several studies have provided estimates of Chiari malformation prevalence (0.01–3.6% of the population), these studies are based on imaging data collected at a single center or hospital and may not reflect the true prevalence of the condition [148, 149]. Nevertheless, these studies are valuable in describing the epidemiology of Chiari malformations. For example, Meadows and colleagues [150] conducted a retrospective examination of more than 22,000 brain MRI scans and estimated the prevalence of CMI at 7.8 per 10,000. The earlier studies from Western countries reported prevalence estimates of 8.2–8.4 per 100,000 [151, 152]. One study based on 2 years of data for newborns admitted to a hospital in Pakistan reported that 3% of all the cases were diagnosed with Chiari malformation [153].

Lee and colleagues [154] retrospectively reviewed 21 years of medical records for pediatric patients who underwent surgery at an institution in Korea for symptomatic CMI. A total of 54 children were identified with symptomatic CMI. Four patients were between the ages of 3 and 27 months, 9 were 3 and 4 years of age, and 41 were 5 and 17 years of age. More males than females were identified in the younger two age groups, but more females than males were identified in the oldest age group. Sakushima and colleagues [155] conducted a survey of hospitals in Japan between August 2008 and July 2009 and found that among a sample of 708 patients with syringomyelia, 48% were diagnosed with CMI and 8.1% with CMII. The authors also reported that Chiari malformation was more common in children than adults.

A few studies examined incidence of Chiari malformations. In one study, 3 years of ultrasound examinations for 22,500 pregnant women from East Azarbaijan in Iran were reviewed to estimate incidence of these conditions [156]. Of the 22,500 pregnancies, 112 (or 0.5%) of fetuses had central nervous system anomalies and 41 had Chiari malformations. Ghavami and Abedinzadeh [156] concluded that Chiari malformations and hydrocephalus were the two most common central nervous system abnormalities in East Azarbaijan in Iran.

While the exact cause of Chiari malformation is unknown, research suggests that genetic factors are the most likely. For example, Schanker and colleagues [149] described a series of three family pairs with CMI and suggested that along with the previously described underlying culprit genes, estrogen may also be a factor in the development of Chiari malformations. CMI has also been found to coexist with ASD, but CMI is often under-recognized in individuals with ASD because symptoms are attributed to autism [157].

Tectocerebellar Dysraphia

Tectocerebellar dysraphia is an extremely rare congenital malformation consisting of vermian hypoplasia or aplasia, an occipital encephalocele, and dorsal traction of the brain stem, such that the hypoplastic cerebellar hemispheres are rotated around the brain stem to lie ventrolaterally [158]. Very few cases of tectocerebellar dysraphia have been reported in the scientific literature [159]. Children with tectocerebellar dysraphia generally have very low intellectual functioning, and 40–75% die before their first birthday, largely because of hydrocephalus [160].

Tectocerebellar dysraphia is a condition so rare that prevalence and incidence estimates cannot and have not been made. Thus, we examined eight published case reports; these case reports describe the following cases: one 3-month-old boy in India [161], one 5-month-old girl [162], and two cases (8-year-old boy, 2-month-old boy) but the authors also noted three other cases previously described [163], 7-month-old male in India [158], 4-year-old girl in the United States [164], and four cases (three girls, one boy) in Saudi Arabia [165]. Some authors suggest that variants of tectocerebellar dysraphia (e.g., tectocerebellar dysraphism with an occipital encephalocele) are structural manifestations of Joubert syndrome [164].

Other

Cerebellitis

Acute cerebellitis is an inflammatory syndrome characterized by cerebellar dysfunction ([166], as cited in [167]). Vomiting, headaches, tremors, nystagmus, dysarthria, and states of consciousness ranging from sleepiness to coma are common symptoms of severe cerebellitis [168, 169]. Patients with acute cerebellitis may also exhibit broad-based gait disturbance, poor coordination of finger-to-nose movements (dysmetry), and irritability [168]. Cerebellitis typically occurs in early childhood either during or after infection or postvaccination or has autoimmune etiologies.

An important causative pathogen for cerebellitis is varicella zoster virus (VZV), an acute, exanthematous, and highly infectious disease, which causes chickenpox (varicella) in childhood, and shingles (herpes zoster) in later life [168, 170]. In a retrospective study using 10 years of data (October 2003–June 2013) from Bambino Gesu Hospital, Rome, Italy, Bozzola and colleagues [168] found that 48 out of 457 (10.5%) children hospitalized with varicella developed acute cerebellitis. All children were unvaccinated for the virus. The highest frequency of cerebellitis occurred in children aged 1–5 years (60.9%), followed by children aged 5–10 years (34.1%), and those 10+years (5%). Girls and boys were affected equally. See Table 5 for a summary of study statistics.

The majority of the literature describes isolated case reports of the most severe but rare cases of cerebellitis (cf [171]), and these cases are typically associated with viruses other than VZV. Specifically, cases of acute cerebellitis have been associated with the Epstein-Barr virus, mycoplasma pneumoniae, rotavirus, human herpesvirus 7, mumps, influenza, and nonspecific viral infections (see [171] for a review). Hackett and colleagues [172] recently reported a case of a 6-year-old girl with an influenza A (H1N1) infection in Ireland presented with acute cerebellitis. In the United States, Hashemi and colleagues [173] described the first reported case of a 9-year-old boy, who presented with hemorrhagic cerebellitis secondary to *Plasmodium falciparum* infection, after traveling in Tanzania. In their retrospective evaluation of the medical records of 194 patients with Epstein-Barr virus infection who were hospitalized in the Department of Infectious Diseases and Child Neurology at the University of Medical Sciences in Poznan, Poland, between January 2010 and January 2015, Mazur-Melewska and colleagues [174] found two cases of cerebellitis (1.03%). Uchizono, Iwasa, Toyoda, Takahashi, and Komada [175] reported what may be the first case of a 7-year-old girl presenting with cerebellitis following group A streptococcal infection in Japan. Although no genetic causes have been identified for acute cerebellitis, Xu and colleagues [176] reported its occurrence in identical twin boys (aged 15 years) 8 days apart in Shijiazhuang, China; a viral infection, however, could not be ruled out. An important challenge for physicians and epidemiologists is to correctly identify the acute cerebellitis because there is considerable overlap in presentation with acute post-infectious ataxia [28] and opsoclonus-myoclonus syndrome [177].

Cerebellar Stroke

Cerebellar stroke is characterized by complaints of dizziness, vertigo, and vomiting [178]. Pontine compression and acute hydrocephalus secondary to the obstruction of the fourth ventricle may occur as a result of swelling after the infarction, which may further result in decreased level of consciousness and arousal. Cerebellar injury early in life stunts cerebellar growth and negatively affects neurodevelopment (cf [179]).

As is true of other cerebellar disorders, cerebellar stroke is unusual in children (see [180]). When cerebellar infarction does occur, these cases are described in the research literature (see Table 5). For example, Lin and colleagues [180] reported a case of a 12-year-old boy presenting with vomiting, gait disturbance, and headache; cerebellar stroke was confirmed with magnetic resonance angiography. Interestingly, the boy had no history of neck manipulation, trauma, or other relevant medical history. In their retrospective evaluation of 977 childhood (<16 years of age) cases of malaria in England and Wales reported between January 2004 and December 2008, Garbash and colleagues [181] found that one child developed cerebellar infarction. Estimating incidence and prevalence has been described as difficult because symptoms, such as ataxia, are not seen clearly during a bedside examination [178]. The overall incidence of cerebellar stroke across all ages is estimated to be 1.5% (M_{age} = 65 years) (see [182]). Thakkar and colleagues [43] reported that of the 120 cases of acute ataxia that occurred in children (0–18 years of age) that were seen at Children's Hospital of Pittsburgh between January 2003 and December 2013, cerebellar stroke was identified in two (1.7%) cases. See Table 5.

Risk factors for cerebellar stroke typically include trauma, drugs, and central nervous system infection (see [183]). When trauma has been sustained through sport, stroke may occur in boys 6.6 times more than in girls [183] and may occur with sudden movement [184]. Other risk factors include congenital cervical anomaly and vascular or connective tissue disease. After reviewing pediatric cases of vertebral artery dissection (VAD) described in the literature, Hasan and colleagues [183] reported a high incidence of associated cervical anomalies (i.e., 10/68 cases). Although rare, cerebellar stroke may occur in children and young adults who overdose on tricyclic antidepressants [185]. Thoon and Chan [186] have also reported one case of stroke in the left cerebellum in a 10-year-old girl following influenza vaccination during influenza season. Another important risk factor for cerebellar infarction is prematurity [179, 182]. Khair and colleagues [182] described a case of a 4.5-year-old girl, who was one member of a quadruplet born at 28 weeks gestation, presenting with a number of symptoms indicative of cerebellar stroke. Cerebellar infarction was subsequently confirmed with an MRI. Cerebellar injury is important to identify as it has important implications for long-term cognitive development [179].

Table 5 Prevalence, incidence, and/or number of cases reported in studies of investigating other cerebellar conditions

	Study details				Population					
Disorder Author, date	Publication type, research design	Country/ region	Study period	Data source	Age	Sex	*N*	Prevalence	Incidence	Number of cases or families
Cerebellitis										
Hackett et al., 2013	Case report	Ireland	–	–	6 years	F	1	–	–	1 case associated with influenza A
Hashemi et al., 2015	Case report	US	–	–	9 years	M	1	–	–	1 case associated with *Plasmodium falciparum* infection
Mazur-Melewska et al., 2015	Retrospective evaluation, case report	Poland	2010–2015	University of Medical Sciences	5.1 years	84F; 110M	194 with Epstein-Bar Virus	–	–	2 cases (1.03%)
Uchizono et al., 2013	Case report	Japan	–	–	7 years	F	–	–	–	1 case associated with group A streptococcal infection
Xu et al., 2008	Case report	China	–	Third Hospital, Hebei Medical University	15 years	2M	–	–	–	2 cases likely associated with a viral infection

Cerebellar stroke										
Garbash et al., 2010	Retrospective	UK (England, Wales)	January 2004–December 2008	Pediatric Intensive Care Unit Audit Network (PICANet)	Children (<16 years)	–	977 malaria cases	–	–	1 case of cerebellar stroke
Kawakami et al., 2009	Case report	Japan	–	–	8 years	M	1	–	–	1 case associated with trauma during sport
Khair et al., 2014	Review, case report	Qatar	–	–	4.5 years	F	1	–	1.5%	1 case, premature birth
Lin et al., 2007	Case report	Taiwan	–	–	12 years	M	1	–	–	1 case
Thakkar et al., 2016	Original research, retrospective	USA (Pittsburgh)	January 2003–December 2013	–	0–18 years	–	120	1.7% among cases of ataxia	–	2 cases
Thoon and Chan, 2012	Case report	Chinese-Thai	–	–	10 years	F	1	–	–	1 case associated with influenza

Conclusions

For many cerebellar disorders, prevalence and incidence rates are unknown, or the values have been underestimated; this is true both at the global and regional levels. Scant epidemiological information can be partly attributed to lack of comprehensive healthcare systems in various parts of the world (see [27]), making diagnosis at an early age difficult or impossible. Fetal loss may also contribute to inaccurate epidemiologic measure, because prevalence and incidence are typically estimated using living individuals [187]. Underestimates may also be the result of cases of cerebellar disorder not being classified accurately in published studies; as such, they may be excluded from analysis (see [30]). In a similar vein, in an effort to include a greater number of affected individuals in epidemiological studies, groups of patients may be relatively heterogeneous in composition (see [30]). Thus, case studies become a very important means with which to communicate the various signs, symptoms, comorbidities, and complications associated with a certain disorders, particularly for those cerebellar disorders that have been described as extremely rare (e.g., cerebellar agenesis, tectocerebellar dysraphia). Further population-based epidemiological studies are important for determining the impact of cerebellar disorders worldwide and providing information regarding the causes and appropriate treatments for these disorders.

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Cerebellar Transplantation: A Potential Model to Study Repair and Development of Neurons and Circuits in the Cerebellum

Constantino Sotelo

Abstract Neuronal transplantation offers the advantages of a unique experimental situation that allows the in vivo study of cell-to-cell interactions between embryonic and adult neural partners. This approach was developed to study the possibility to replace missing neurons in pathological situations. In our model, the cerebellum with spontaneous mutations, *Purkinje cell degeneration, nervous, Lurcher* (*pcd, nr, Lc)* affecting Purkinje cells (PCs), this substitution occurs. Embryonic PCs can trigger in adult Bergmann fibers molecular changes required for migration and ultimate synaptic integration of the former, although this integration is not complete because the full contingent of efferent projections failed to establish. The grafting approach evolved as a suitable tool that, through heterotopic and heterochronic transplants, allowed the investigation of the role of cellular and molecular microenvironment on the acquisition of neuronal phenotypes and on the differential ability to regenerate amputated axons of specific populations of central neurons. Finally, new approaches developed in the twenty-first century, with the advent of stem cells and cell reprogramming, are mentioned and some of the earliest cerebellar trials with these pluripotent cells discussed.

Keywords Transplants • Embryonic and adult cell interactions • Neuronal replacement • Axon regeneration • Stem cells • Lineage reprogramming

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Introduction and History

The Neuron Doctrine: Plasticity of Adult Circuits, but Absence of Neuronal Regeneration

Until recently, neuroscientists of my generation, whose interest in the nervous system began long ago, have been believing in the dogmatic but erroneous concept that in the central nervous system (CNS) there is no further possibility for neuronal proliferation after the end of the constructive period of brain development. This notion was developed at the end of the nineteenth century and beginning of the twentieth by researchers working on brain development [1–3] or interested in the principles of pathology based upon the regenerative and proliferative potential of body cells [4]. Ramón y Cajal [3] summarized this concept of the adult nervous system best in his famous statement: "In adult brains, nerve pathways are something fixed, ended, immutable. Everything may die, nothing can regenerate," a pessimistic concept that foreshadows the fate of many neurological disorders related to the aging process.

The hope for a peaceful physiological aging became even more elusive at the beginning of the second half of the twentieth century, when Harold Brody [5] morphometrically analyzed human brains from birth to the age of 95 years old. Brody [5] concluded that, based on volume shrinkage, determinations correlated with cell counts; from the age of 21 onward, there was a progressive neuronal loss ranging in magnitude depending on the analyzed cortical areas. Therefore, not only our adult brain was unable to proliferate but, even worse, it started losing neurons early on. John Eccles [6] reflected on this dire situation: "Soon after birth ceases all generation of neurons. Thereafter neuronal death takes over." All these arguments compelled us to assume that age-related loss of neurons and its subsequent decline in brain function were unavoidable. With the arrival of modern and more accurate imaging (MRI) and morphometric methods (modern stereology), it became evident that the results mentioned earlier were due to technical limitations and that neuronal cell death was not as pronounced as supposed. In fact, Herbert Haug et al. [7, 8] were able to show that there was virtually no loss of neurons before age 60, and even then, the progress of neuron death was slow and uneven across the various brain regions. This makes a big difference in respect to neuronal loss between normal aging and neurodegenerative diseases, particularly Alzheimer's disease [9]. Nevertheless, the controversy has not been fully resolved. Thus, it was recently proposed that "some aspects of age-related cognitive decline begin in healthy educated adults when they are in their 20s and 30s" [10] and that although this decline is not necessarily accompanied by neuronal death, dendritic alterations, especially loss of spines by reducing the number of synapses, could be its cause [11].

Finally, the apparent lack of neuronal proliferation did not stop scientists from deeming the adult brain as a changeable organ. In his opera magna, *Texture of the Nervous System of Man and the Vertebrates*, Ramón y Cajal [12] clearly summarized the concept developed by Alexander Bain [13] and known today as "morphological neuronal plasticity." Eugenio Tanzi [14] and Ernesto Lugaro [15],

followers of Ramón y Cajal's neuron doctrine, specifically formulated it naming synapses as the preferred place for plastic changes (see in [16]). These theoretical notions have evolved today so that it could be possible to accept the concept proposed by Tanzi that the nervous system is a "neoteny," because some developmental features are preserved in adulthood (for further details, see the recent history of the morphological plasticity in [17]).

Changing Bizzozero's Classification: Not All Neurons Are Perennial Cells. The Discovery of the Existence of Adult Neurogenesis and Neural Stem Cells Even in Mammalian Brains

The only drawback, but crucial in the history of "neoteny," has been neuroscientists' resistance to accept the possibility that even a mild neuronal proliferation could occur in adult mammalian brains. The development of the autoradiographic method for labeling cell divisions with tritiated thymidine greatly advanced the search for neuronal division [18], already conceived by Allen [19]. It quickly became clear that adult neurogenesis was possible in cold-blooded vertebrates (fish, amphibians, and reptiles) but inexistent in mammals. It was Joseph Altman [20, 21] who foresaw the possibility of the existence of adult neurogenesis, at least in the granule cells of the hippocampal dentate gyrus. Despite the later ultrastructural analysis corroborating the neuronal nature of the labeled cells [22], the traditional dogma that the generation of new neurons in the brains of grown-up warm-blooded animals did not exist has persisted for almost another decade. Indeed, it was only toward the end of the twentieth century that Brent Reynolds and Samuel Weiss [23] provided irrefutable evidence of adult neurogenesis and of the presence of neural stem cells in adult mouse CNS.

One of the early issues to be solved was to determine whether the existence of neurogenesis in the adult mammalian brain was just a vestige of the phylogenetic evolution or played some important physiological role. Experiments carried out in the two centers where adult neurogenesis is more effective, the dentate gyrus of the hippocampus [24] and the subventricular zone at the origin of the rostral migratory stream (RMS) to the olfactory bulb [25, 26], showed that delayed neurogenesis exerted an important function, closely correlated with a respective increase or decrease in either spatial (dentate gyrus) [27] or olfactory memory (olfactory bulb) [28].

Neuronal Transplantation

Neuronal Replacement in "Point-to-Point" Cerebellar Circuits

It is first important to remember that in addition to the majority circuits wired in a "point-to-point" manner as the cerebellum, the brain is also formed by a second class of systems we named "global" [29]. The latter are formed by monoaminergic

and peptidergic modulatory systems that can function without morphological synaptic junctions, through paracrine release of neurotransmitter [30] diffusing into the extracellular space to exert its inhibitory or excitatory action on nearby receivers equipped with specific receptors. The conditions that should be fulfilled for successful neuronal replacement in the cerebellum are therefore much more difficult to achieve than in "global systems" where most of the work in neural grafting for therapeutic purposes (Parkinson disease) has been carried out [31]. In cerebellar transplants, the grafted neurons replacing the missing ones have to reach their normal location, complete their synaptic integration with specific host afferents, and provide efferent axons able to appropriately find distant postsynaptic elements of the host, allowing a mirror reconstruction of the normal cerebellar connectivity. In this section, only PCs will be considered. The results obtained with transplantation of molecular layer interneurons have been recently reviewed [32], and those regarding granule cells are discussed in the third part of this chapter ("Cerebellar transplantation of granule cells").

Positive Results in Favor of the PC Replacement in Mutant Mice with Heredodegenerative Ataxia

Morphologic Results

The circuitry of the cerebellar cortex, as reported by Santiago Ramón y Cajal [33], is relatively simple: two main extracerebellar afferent systems, the climbing fibers and the mossy fibers (CFs and MFs), convey their information either directly (CFs) or through the granule cells (MFs) to the Purkinje cells, the pivotal element and sole output neurons of this cortex, which in turn transfer the processed information to the deep cerebellar nuclei (DCN). In addition, these convergent and divergent excitatory inputs reaching each PC are balanced by the inhibitory action of the GABAergic interneurons, mainly the Golgi cells in the granular layer, and the molecular layer interneurons. Given the pivotal role of the PCs, it is obvious that their loss would provoke a severe ataxia that will persist if they are not replaced by new neurons of a similar nature. The cerebellum appears, therefore, as a privileged neural center where to test the reparative ability of grafts of embryonic neurons in "point-to-point" systems. The selected model for heredodegenerative ataxia was the mouse carrying the "Purkinje cell degeneration" (pcd/pcd) mutation [34]. *pcd* is an autosomal mutation that affects the gene Nna1 [35]. This gene codes a new ATP/GTP-binding protein related to zinc carboxypeptidases. It is an interesting protein involved in regenerative as well as degenerative events, previously identified by its induction in motoneurons during axon regeneration [36]. Afterward, although we will not discuss the results here, we have corroborated the observations in *pcd/pcd* mice using as host cerebella *nervous (nr/nr)* [37] and *Lurcher* (*Lc*/+) mutant mice [38] also provoking degeneration of PCs although these mutations took place in completely different genes. Since the results were similar in each mutant, we could

conclude that they were independent of the affected genetic locus and the genetic background of the cerebella of the three different mutants used.

In *pcd/pcd* mice, the cerebellum at birth is morphologically normal, and it is only toward P16–P18 that some PCs start degenerating. Three to four weeks later, more than 99 % of this neuronal population has died [34]. Counts of the number of PCs (the only Calbindin-positive cells, CaBP+, of the cerebellum) showed that a maximum of 110 cells survived in an adult (P60) *pcd* cerebellum and almost all of them in lobule X, that is to say less than one per thousand [39]. This degeneration, followed by apoptotic death (see in [40]), provoked a severe ataxia, which started when the homozygous mice was 25 days old. The ataxia was worsened by the severe transsynaptic atrophy of the PCs' presynaptic partners, particularly the inferior olivary neurons, whose number progressively dropped, and that in 300-day-old mutants almost half of them had disappeared [41]. These retrograde changes were accompanied by a drastic atrophy of the remaining target-deprived CFs, which showed monoplanar atrophic arbors, with sparse varicosities and a few round-shaped boutons radiating in the molecular layer [42, 43]. Moreover, the number of parallel fibers (PFs) was also diminished in aged *pcd* mutants, a reduction that was correlated with an important retrograde death of granule cells [44]. In 1-year-old *pcd/pcd* cerebella basket cell axons also seemed to severely reduce in number [45], indicating that a large proportion of the neurons monosynaptically connected to the dying PCs was affected by retrograde transsynaptic death. Nevertheless, in the 50- to 60-day-old mutants used in our grafting experiments, only a few weeks after the disappearance of the Purkinje cells, the vast majority of the different classes of presynaptic fibers, although slightly atrophic, were still present in the cortical neuropil, a prerequisite for the transplanted neurons' successful synaptic integration.

The severe ataxia of 25–50-day old *pcd/pcd* mutants helped identify and select them for the grafting experiments. Two types of transplants were used: cell suspensions of E12 embryonic cerebella taken from isogenic embryos and small pieces (less than 1 mm^3) of E12 cerebellar anlagen, what we called solid grafts. The latter produced a much better yield, and most of the transplantations were done with solid grafts [46]. To search for synaptic integration of grafted PCs in the adult mutant cerebellum, 1–2 months after the grafting, the host cerebella were fixed and immunostained with an anti-CaBP antibody, or embedded in Araldite for ultrastructural study. In addition, some grafted mice were used for electrophysiological studies [47]. Due to the total depletion of PCs in most dorsal lobules where the grafts were placed, the CaBP-stained cells belonged exclusively to grafted PCs. The latter always occupied an ectopic position because their cell bodies never reached the interface between molecular and granular layers (Fig. 1b). The dendritic trees of the grafted PCs, although flattened in the plane perpendicular to the host PFs (Fig. 1b), were atypical and exhibited two or more stem dendrites emerging not only from the apical soma but from lateral and even basal regions as well. Secondary branches, provided with distal spiny branchlets studded with spines, emerged from the stem dendrites (Fig. 1b). The grafted PCs expanded over two and a half folia (Fig. 1a) and received

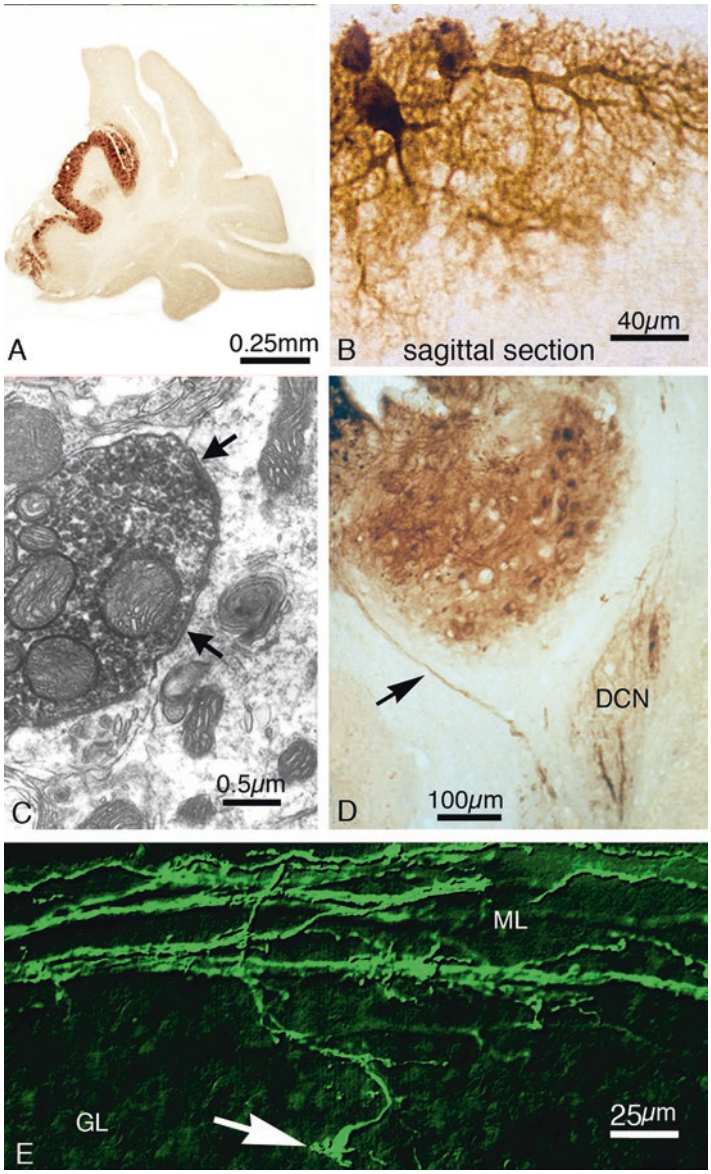

Fig. 1 Micrographs of the cerebellum of adult *pcd/pcd* mice immunostained for calbindin 2–3 months after grafting. (**a**) Low magnification of the grafted PCs that have migrated into the host cortex. The *asterisk* marks the graft remnant. (**b**) Sagittal section through the vermal cortex, illustrating the flattened shape of the dendritic tree of grafted PCs. (**c**) Electron micrograph of the medial deep cerebellar nucleus. The immunolabeled axon terminal of a grafted PC is synapsing (*arrows*) on the cell body of a deep cerebellar nuclear neuron. (**d**) Graft remnant in the host white matter and adjacent cortex. The *arrow* points to a thin fascicle of PC axons in their way to the host deep cerebellar nuclei (DCN). (**e**) Immunofluorescence of calbindin-positive thin fascicles of grafted PCs axons within the molecular layer (ML) of the host cerebellum. The *arrow* points to an arrested growth cone at its entrance into the granular layer (GL)

a normal spatially distributed contingent of presynaptic inputs. Thus, PFs synapsed mainly upon PC long-necked spines arising from narrow distal branches (Fig. 2a), while CFs did it over thorns emerging from thicker dendrites (Fig. 2a), as in normal cerebellum. However, the axons of the host basket cells never formed "pinceaux" around the initial segments of the axons of the ectopically located PCs, although both elements could establish synaptic connections (Fig. 2b). This synaptic abnormality was systematically observed for all ectopic PCs studied whatever the mutation or the situation analyzed: *weaver* and *reeler* cerebella [48, 49], transgenic mice with plexin B2 knockout [50]. This lead to the conclusion that the abnormality was not caused by the transplantation itself but that the presence of the PC axon initial segment at the interface between granular and molecular layers is a prerequisite for the establishment of "pinceaux" formations. Therefore, from a morphological viewpoint, despite the important synaptic failures reported above, it was concluded that the grafted PCs were synaptically integrated in the cortical circuit of the host cerebellum and that the target-deprived host axons could recuperated their synaptic affinity and normal size when innervating the newly added PCs. Another important failure was the rarity of PC axons able to cross the underlying granule cells, a very important feature to reach the white matter and the deep cerebellar nuclei (see "The difficulties to restore corticonuclear projections argue against the possibility of succeeding complete PC replacement by embryonic cerebellar transplants").

Electrophysiological Results

In collaboration with Francis Crepel and Robert Gardette [47], we studied the electrophysiology of the grafted PCs. Using in vitro slices of *pcd/pcd*-transplanted cerebella, PCs were impaled with intracellular microelectrodes, and their bioelectrical properties, as well as their synaptic interactions, were analyzed by electrical stimulation of the white matter at the base of the folium for the anterograde activation of host CFs and MFs. The study demonstrated first that the grafted PCs had normal bioelectrical properties including sodium and calcium membrane conductances and inward rectification. Moreover, the vast majority of them, 54 out of 55, did not respond to white matter stimulation by antidromic spikes, in accordance with the rarity of PC axons in the granular layer and white matter announced above. Nevertheless, all grafted Purkinje cells responded to electrical white matter stimulation with a typical all-or-none CF (complex spikes) response or complex spike followed by simple spikes. These disynaptic responses (MF – PF activation) were found less frequently because the large amplitude and duration of the CF responses together with their short latency usually masked the eventual consecutive excitatory postsynaptic potentials via MFs and PFs. Finally, inhibitory postsynaptic potentials, like normally connected PCs, were also recorded, corroborating the integration of the grafted neurons in the circuitry of the cerebellar cortex of the host [47].

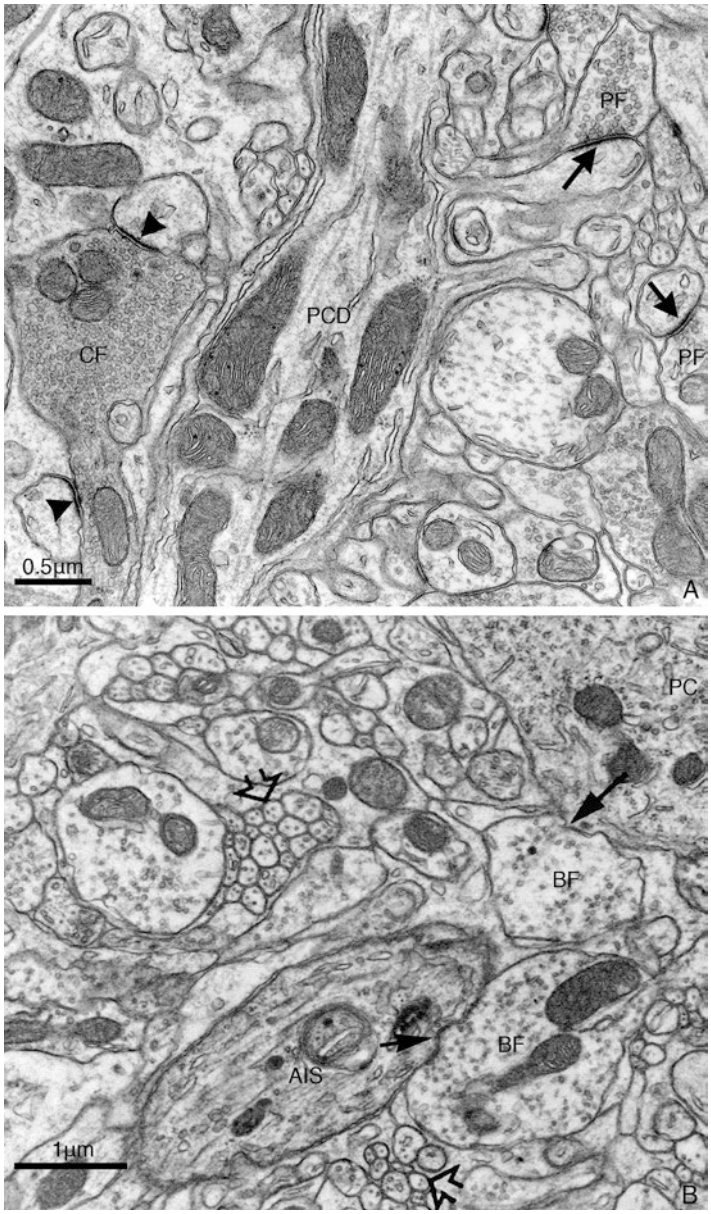

Fig. 2 Electron micrographs illustrating the synaptic input of grafted PCs. (**a**) Parallel fibers (PF) synapsing on spines (*arrows*) of a PC dendrite (PCD). A nearby climbing fiber (CF) synapses on two other PC spines (*arrow heads*). (**b**) Initial segment of the axon (AIS) of an ectopically located PC body (PC) among fascicles of parallel fiber (*open arrows*). Both AIS and perikaryon are synaptically contacted by axon terminals of molecular layer interneurons (*arrows*), but "pinceau formation" is missing

Parasagittal Compartmentation of Grafted PCs

One of the special features of PCs is their biochemical heterogeneity, underlining the subdivision of the cerebellum in parasagittal modules, that defines its anatomical and functional organization [51]. This biochemical heterogeneity, first detected by the asynchronous expression during early development of markers that in adulthood are present in all PCs [52], maintains the same topography later in development when the selective markers of adult heterogeneity begin to be expressed [53]. This continuity makes PCs the prime organizer of the extracerebellar afferent projections [54]. A PC marker of these modular compartments is the zebrin-1 molecule, which has allowed for the analysis of the possible development of a zonal organization within grafted PCs [55]. The study was carried out in adult rat's grafted cerebellum pretreated with intraparenchymal kainic acid injections to produce necrosis and death of affected PCs. Modular organization was searched for either in the graft remnant itself or within those PCs that migrated to be incorporated into the host cortical circuit. Immunohistochemistry with zebrin-1 antibodies revealed with HRP was used to identify the alternating microzones with zebrin-1-positive and zebrin-1-negative PCs, and immunofluorescence of CaBP was used to visualize those zebrin-1-negative PCs, the only ones to be visible due to the quenching of fluorescence by the diaminobenzidine precipitate of their zebrin-1 immunostaining. In both instances, the existence of alternating zebrin+ and zebrin– microzones was detected. In the graft remnant, the alternating clusters contained up to 10 PCs (Fig. 3a), whereas in the host parenchyma invaded by grafted PCs, the bands were formed by only one to three PCs by section plane (Fig. 3b), indicating that PCs might have genomic heterogeneity and could reach their predetermined fate even in an adult environment. These bands did not correlate in distribution nor size with host bands.

In collaboration with Richard Hawkes [56], we investigated, also using transplants, if the micro-zonation of PCs during development was due to intrinsic molecular differences between PC progenitors or was just the result of receiving presynaptic inputs, particularly from the host olivo-cerebellar projection. This might be the case for transplants in adult rat cerebellum after kainic acid injection where, despite the loss of PCs, the CFs although atrophic were maintained [42]. The approach was to isolate the cerebellar anlagen from the specific incoming afferent fibers, before the age of initiation of the synaptogenesis between PCs and CFs or transiently with MFs [57]. To this aim, solid grafts of E12 rat cerebellum were transplanted to either a cavity in the neocortex of adult rats (in cortico) (Fig. 3c, d), or in the anterior chamber of the eye (in oculo). The grafts were able to reach maturity without being exposed to the action of CFs and/or MFs. In both these types of transplants alternating clusters of zebrin-1+ and zebrin-1– PCs developed without the influence of neither CFs nor MFs, pointing to the intrinsic nature of the biochemical heterogeneity of PCs. This result was extremely useful to formulate the hypothesis that the topography of the projections in the cerebellar cortex was regulated by PCs [54], which not only are the pivotal elements in transmitting functional information from the cortex to the DCN but also orchestrate the developmental organization of the cerebellum. It can be concluded that without PCs there is no cerebellum.

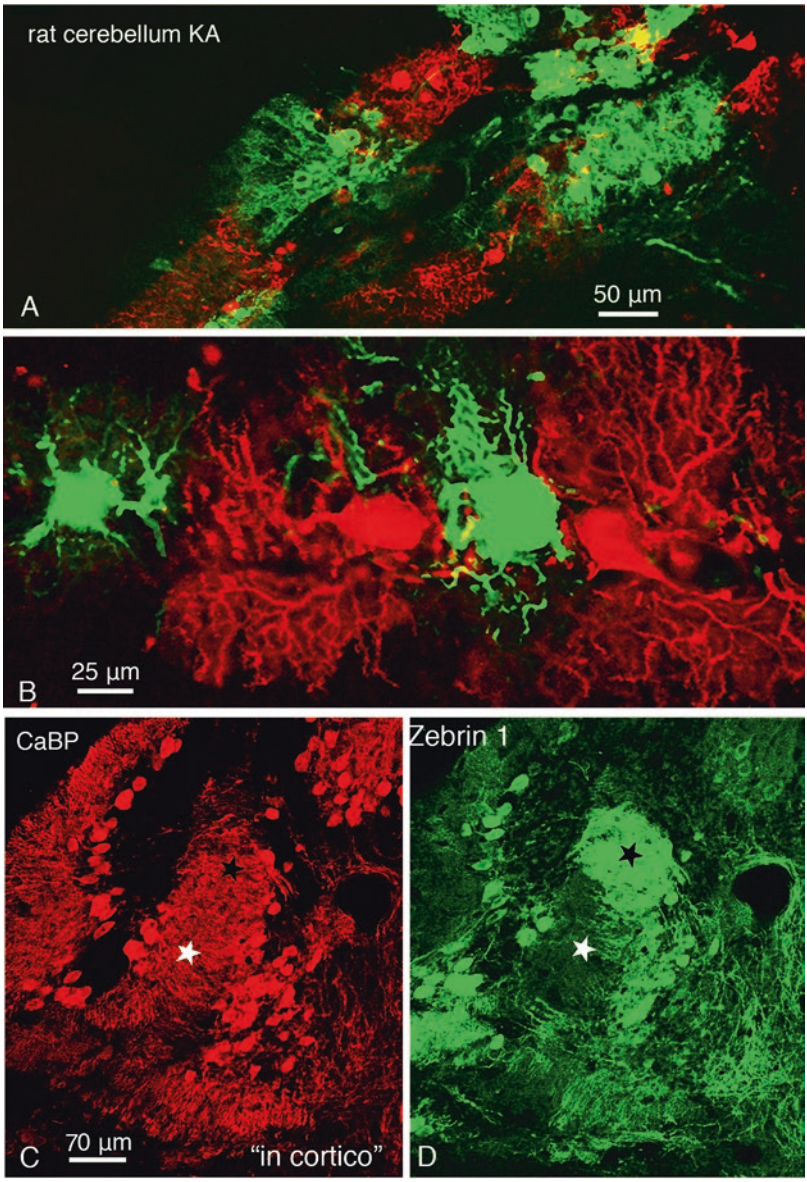

Fig. 3 (**a**, **b**) Two color stain of graft derived from rat suspension of E15 cerebellar anlage into rat cerebella pretreated with intraparenchymal kainic acid injections. Note the extensive integration of PCs into the rat host PC-deficient molecular layer revealing compartmentalization into zebrin-I + and – areas. The anti-zebrin-I was peroxidase stain converted into *green color* with Photoshop CS4, whereas the *red color* shows fluorescent stain for CaBP. (**c**, **d**) Double immunofluorescence of an E13 cerebellar anlage grafted 2 months before into a cavity in adult rat cerebral cortex. Note that while all PC are calbindin positive (rhodamine), only part of them are Zebrin-I positive (fluorescein) revealing alternative clusters of zebrin-I – (black star) and zebrin-I + (white star) PCs

The Difficulties to Restore Corticonuclear Projections Argue Against the Possibility of Succeeding Complete PC Replacement by Embryonic Cerebellar Transplants

Finally, the most negative part of the study focused on the fate of grafted PC axons and their efferent projections that cannot establish proper connections with their faraway host targets. As stated above, most of the axons of the grafted PCs remained within the host molecular layer, without reaching the white matter, as if they could not overpass the non-permissive territory offered by the underlying granular layer, a barrier missing during the developmental period during which these axons normally reach the prospective white matter in their way toward the deep cerebellar nuclei (Fig. 1e). The electrophysiological analysis corroborated this morphologic result since only one of the 55 grafted PCs impaled had antidromic potentials after white matter stimulation [47]. Once in a while, a few CaBP+ axons ran within the white matter on their way to the DCN. In most of these cases, PC somata were present in the granule cell layer or even in the white matter. Axons from these ectopic neurons can form thin fascicles that can reach the DCN even after covering relatively long distances (Fig. 1d). The defasciculation of these thin fascicles took place once they arrive to the DCN, where they gave rise to calbindin immunostained dots resembling axon terminals. Their actual nature was corroborated by electron microscopy immunocytochemistry (Fig. 1c). Even more rarely, some of the Purkinje somata located within the host molecular layer, and therefore synaptically integrated, was in continuity with underlying clusters of grafted embryonic bridges. In these situations, their axons can grow through the bridges into the white matter reaching the DCN. These observations provided evidence emphasizing that distance was not the main obstacle for the correct growth of PC axons but that the presence of the non-permissive environment of the host granular layer was. To overcome this obstacle, new grafts were prepared by placing tiny solid pieces of E12 cerebellar primordium in a cannula and implanting them deep in the cerebellar parenchyma of the host to establish a bridge between cortex and DCN that could serve as a permissive passage for axons of grafted PCs that have colonized the host molecular layer, rebuilding this way a new corticonuclear projection [58]. Although technically correct, the obtained yield was very poor, since only a few grafted PCs cortically integrated found their way to the target through the bridge (DCN).

Of course, the problem is solved if the transplantation is performed in utero during embryonic life, as done by Ferdinando Rossi and collaborators [59]. For identification of grafted cells, donor cells were dissected from β-actin-enhanced green fluorescent protein (EGFP) transgenic rats (E14) and transplanted as a single-cell suspension in the fourth ventricle of E16 rat embryos. In this case, although the remaining grafted PCs were less numerous because of the competition with the almost isochronic PCs of the host [59], in adulthood their vast majority occupied an orthotopic position at the molecular/granular layer interface, and their axons reached normally their terminal domains in the DCN. However, if the age of the host is progressively increased (P1, P8) the number of orthotopic PCs decreases, from almost 90 % at E16, to 40 % at P1 and at P8 all surviving grafted PCs remain

ectopic. These results emphasize the obstacle provided by the mature granular layer for the growth of PC axons, and the current difficulties to offer cerebellar grafting as a possible way to treat ataxia in mature cerebellum.

Embryonic and Adult Cell Interactions

Despite the indisputable importance of genetic programs for the developmental project of the cerebellum, it is well known that epigenetic factors consecutive to cell-to-cell interactions are also important. In most normal instances, these interactions occur between isochronic cells. Nevertheless, in reparative process as well as during integration of newborn neurons either generated from adult neural stem cells or transplanted from embryos, adult neural cells should interact with immature ones. The first information we gathered regarding these interactions arose from morphological and electrophysiological studies done on adult cerebella transplanted with cerebellar embryonic cells (E12) and analyzed between 3 and 21 days after grafting (DAG) [60–62]. Three DAG, the transplants were already anchored into the host cerebellum, creating a new and ectopic stream of migratory cells at the surface of the affected folia, between the pial basal lamina and the upper surface of the host molecular layer. By 4–5 DAG, a vast band of large neurons entered in a funneling manner in this stream (Fig. 4a) covering large distances, as already discussed up to 2 and 1/2 folia. In preparations immunostained with anti-CaBP antibodies, subpial bipolar PCs were tangentially oriented (tangential migration) (Fig. 4b), and 2 days later, they changed direction to migrate radially, penetrating within the adult molecular layer of the host cerebellum (Fig. 4c). This period of radial migration along Bergmann radial fibers took place between 5 and 8 DAG. Our ultrastructural examination showed that during tangential migration, thin layers of astrocytes surrounded the migrating PCs, whereas during their radial migration, they were apposed to relatively thick stems of Bergmann glia.

Between 11 and 14 DAG, the synaptogenesis between grafted PCs and adult presynaptic axons of the host was very active. CFs translocated from PC somatic spines, where they started synaptogenesis, to proximal branches spines. Simultaneously, PFs and axons of the molecular layer interneurons had established synaptic contacts, respectively, with dendritic spines of the PC distal branches and the shafts of proximal branches. The obtained electrophysiological results completed the morphological ones and revealed that synaptogenesis between host CFs and grafted PCs followed a similar process to that which occurs during development, when both partners were immature. They also went through a transient period of polyinnervation with an average of three distinct CFs synapsing of each PC by 10 DAG, till at least 14 DAG when they become monoinnervated [61].

In conclusion, the establishment of the newly formed connections mimicked very closely the time course and sequence of events happening in normal development. It looks like the embryonic PCs impose a program defined by their own internal clock that leads to their timely ordered synaptic integration [60]. We were

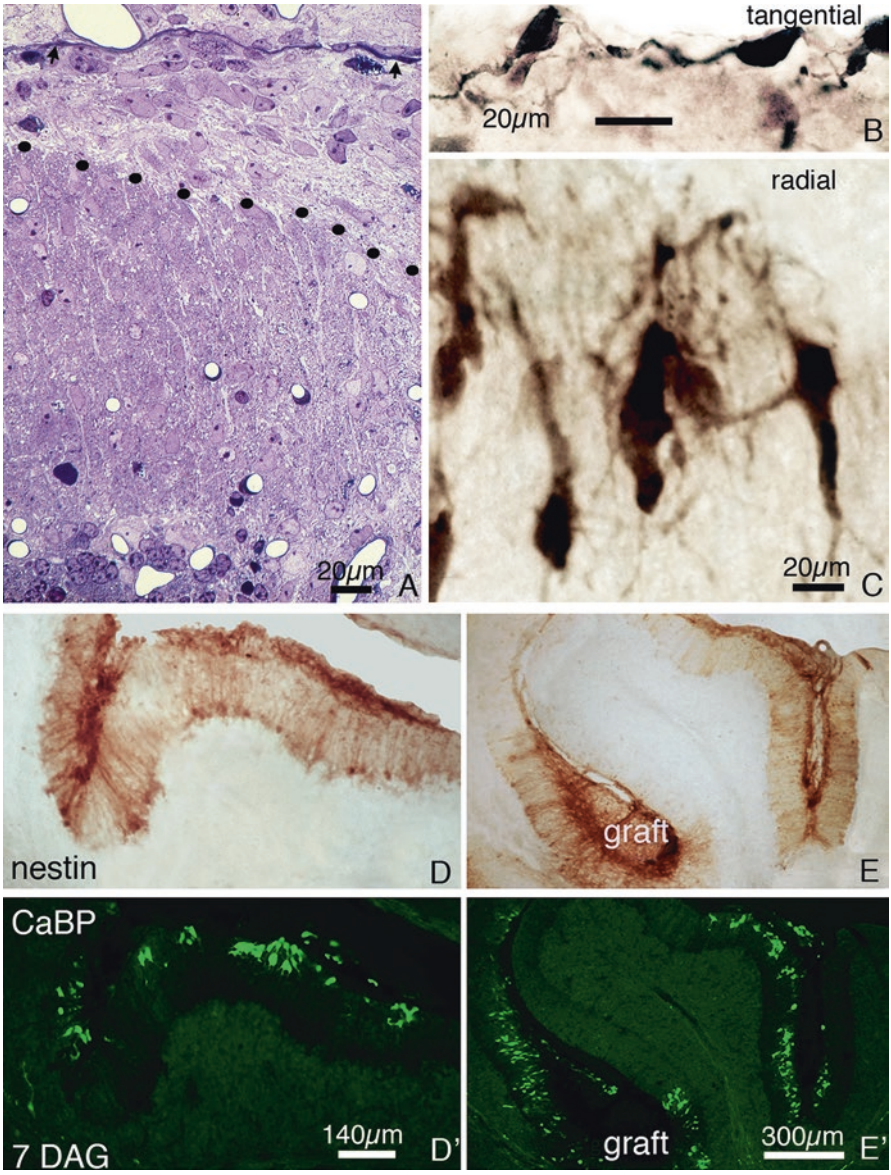

Fig. 4 Development of reciprocal graft-host interactions during PC replacement. Events taking place during the fifth to the eighth day after grafting. (**a**) Between the subpial basal lamina (marked by *two arrows*) and the glial limiting membrane (marked by *large dots*), a funneling stream of tangentially migrating PCs invades the host cerebellum from the graft/host interface (*right* of the micrograph). Micrograph taking from a 1 μm thick plastic section. (**b, c**) Calbindin-labeled PCs, respectively, in tangential (**b**) and radial (**c**) migration. (**d, d', e, e'**) Sagittal sections of the grafted mutant cerebellum double labeled with anti-calbindin (histofluorescence) (**d', e'**) and anti-nestin (immunoperoxidase) (d, e) antibodies to visualize migrating PCs and their glial axes. Note that the adult Bergmann fibers, in the presence of embryonic PCs, change their nestin expression from the null, which characterizes adult animals, to the positive of radial glia necessary for radial migration of grafted PCs

fascinated by these new cyto-sociological rules imposed by the embryonic neural cells, and we wanted to uncover some of the molecular mechanisms governing these embryonic/adult interactions.

Molecular Mechanisms Regulating Grafted PC Migration. Interactions Between Adult Host Bergmann Fibers and Embryonic PCs. New Type of Neural Plasticity: "Adaptive Rejuvenation"

PC migration is a glial-guided migration from the ventricular cerebellar neuroepithelium toward the presumptive cerebellar cortex, to form what is called the PC plate (see in [63]). During ontogenesis it might be thought that expression of the necessary cues by the participating cells is coordinated because they are similar in age. This would not be the case, however, when embryonic neurons are grafted into adult brain. This raises the question as whether the grafted embryonic PCs can induce molecular changes in adult host cells, especially in Bergmann glia, to transiently re-express the molecular cues needed for their migration and synaptic integration into the host [60] or if isochronic embryonic astrocytes leave the graft, acquire within the host parenchyma the radial glia phenotype, and provide the substrate for the migration of grafted PCs.

In order to determine whether either comigration of embryonic PCs and astrocytes or "rejuvenation" of host glia by the embryonic PCs was involved in graft integration, we performed the following experiment [64]. Mutant pcd mice were grafted with cerebellar primordia from homozygous embryos of the transgenic line, Krox20/lacZ14, wherein in the cerebellum, the β-galactosidase activity was detected exclusively on Golgi epithelial cells and their Bergmann fibers, allowing its distinction from the host. Presumptive molecular changes in host Bergmann fibers were investigated by immunohistochemistry with rat mAb-401 antibody (gift Susan Hockfield) [65] that identifies nestin [66] an intermediate filament protein expressed transiently by neural progenitor cells and their glial axes for neuronal migration. This antibody is, therefore, temporary and spatially suited to identify radial glia involved in radial migration [65]. Cerebellar sections of pcd mice that received transplants of Krox-20/lacZ14 transgenic embryos were immunostained either 5 DAG during the tangential migration of grafted PCs, or 7 DAG during their radial migration, or after the end of their complete migration (13 DAG). First, no X-gal-positive cells were found outside the solid graft remnant, a compelling evidence against comigration. Finally, in the folia invaded by grafted PCs, and only during the short period of their radial migration, Bergmann fibers subserving this migration expressed nestin (Fig. 4, d, d', e, e'). This close spatiotemporal correlation allowed us to conclude that a new class of plasticity did occur as a process or "rejuvenation" induced by the grafted embryonic PCs on their adult partners. Therefore, after transient expression of molecular cues associated with PC migra-

tion, adult Bergmann fibers became able to recapitulate the mechanisms employed in normal ontogenesis, enabling migration and synaptic integration leading to the restoration of the disrupted cortical circuitry [60].

Neural Grafting and the Balance Between Neuronal Intrinsic Growth Regulatory Mechanisms and Extrinsic Environmental Stimuli for Central Axon Regeneration

As discussed above, spontaneous recovery of function after mammalian CNS injury is very limited, not only because of the scarceness of adult neurogenesis but mainly because central neurons are unable to regenerate their axons, contrary to what happens in non-mammalian species [67–69], and in mammalian peripheral axons [3]. From the beginning, researchers wanted to know if the distinct behavior of the mammalian central and peripheral neurons was intrinsic to themselves or the result of the different molecular and cellular environment they encountered. Francisco Tello [70], who worked in Ramón y Cajal's laboratory, was one of the first to answer correctly this question. Two distant cuts in peripheral nerves produce aneural nerve fragments, with preservation of their cellular sheaths, Schwann cells, and connective tissue accessories. Such isolated fragments of peripheral nerve were grafted deeply in the neocortex. The nearby cut central axons were then able to develop growth cone-like structures at the distal ends of their proximal stumps that grew for long distances penetrating deeply the grafted aneural nerve. For Ramón y Cajal, these observations clearly indicated that by simply providing the suitable environment, a central axon could regenerate as a peripheral one. Therefore, the study of non-permissive molecules preventing central axonal regeneration has become a research topic of the last 30 years. Thus, proteins of the extracellular matrix at the glial scar, such as cytotactin / tenascin and proteoglycans [71], as well as myelin remnants [72], have been the focus of this research.

Furthermore, these studies showed that the regenerative failure was not solely the result of environmental growth inhibitory molecules but also of central neurons' intrinsic properties. Indeed the latter, after the transitional period of development, loses their ability to reset the required specific set of genes associated with axon growth. For these reasons, it is commonly accepted nowadays that the regenerative process's success depends on the interplay between environmental cues and intrinsic properties of the damaged neurons. It is obvious that both these elements should be taken into account when designing therapeutic strategies for promoting central axon regeneration.

Regarding the cerebellum, the main topic of this chapter, it has been possible to reveal an unusual feature of the adult PCs. They are a rare class of neurons that did not respond to axotomy with neither somatic, retrograde, or degenerative changes (chromatolysis), even when the injury was done close to the axon hillock, as can happen in a few of PCs after transection of cerebellar folia separating the anterior from the posterior vermal lobes, nor dendritic changes [73]. The absence of chromatolysis

was most probably responsible for the lack of regeneration observed on these neurons, because degenerative events seemed needed to activate metabolic and reparative genetic programs required for axon growth [74, 75]. Although PCs did not retract the proximal stumps of their severed axons that remained apposed to the wound cavity for long periods of time, they went through progressive changes characterized by hypertrophy of the recurrent collateral system that gave place to "PCs with arciform axons" (Fig. 5b) [73]. Ramón y Cajal [3] described these changes as a compensatory growth process that transformed PCs from projection neurons into interneurons with short axons. Nevertheless, 3 months after the lesion, a thin and short terminal sprouting appeared, growing little by little up until 18 months, the longest survival time analyzed [76]. After 18 months, the sprouts were numerous and arranged into randomly oriented plexuses, partially filling the regions of granular layer abutting the lesion cavity. These terminal sprouts had established heterotypic synaptic contacts with granular cell dendrites at the glomeruli. These changes observed in the injured axons were spatially and temporally correlated with the cellular and molecular changes occurring in the glial scar. Activated macrophages disappeared much earlier than the initiation of sprouting did. Myelin and its associated neurite growth inhibitory molecules began to decrease 3 months after the lesion. More importantly, some of the reactive astrocytes started at this time to expressed PSA-NCAM, the embryonic form of the neural cell adhesion molecule, changing completely the non-permissive nature of the early glial scar into a permissive substratum for neurite outgrowth. Therefore, the belated axon growth attempt occurred under the form of early thickness increase and late terminal sprouting. The latter occur at the same time as changes in the glial scar. This almost exclusive behavior of PCs to axotomy confers to these neurons the epithet of the central neurons with the poorest spontaneous regenerative capacity.

The behavior of inferior olivary neurons, also axotomized at their distal arbors – CFs – after folia transection, was quite different [77]. They did not become hypertrophic but, instead, became thinner, ending in small terminal bulbs also apposed to the wound cavity. No spontaneous regeneration was observed, although these axons were known by their high plasticity [43]. Contrary to PCs, inferior olivary neurons suffered from a severe retrograde reaction that produced their progressive atrophy [42] and, for many of them, ultimate cell death [78]. Sixty days after axotomy, more than 50 % of olivary cells had died [79].

Early gathered information stipulated that during development, young postmitotic neurons had a much higher capacity for regeneration than mature ones. Furthermore, Oscar Sugar and Ralph W. Gerard [80], using immature rats and following Cajal's method of implanting aneural peripheral nerve fragments, provided a clear demonstration that some regeneration could take place. Based on these facts, many investigators proposed to combine the injury of central regions with simultaneous or delayed filling of the injury track with a large array of grafted biological materials (aneural peripheral nerve fragments, embryonic spinal cord tissue, cultured embryonal tissue, tumor cells or others; see references in Puchala and Windle [81]). This combined approach was extremely useful in revealing the heterogeneity of responses induced by axotomy in different neuronal populations and was used extensively in cerebellar lesions.

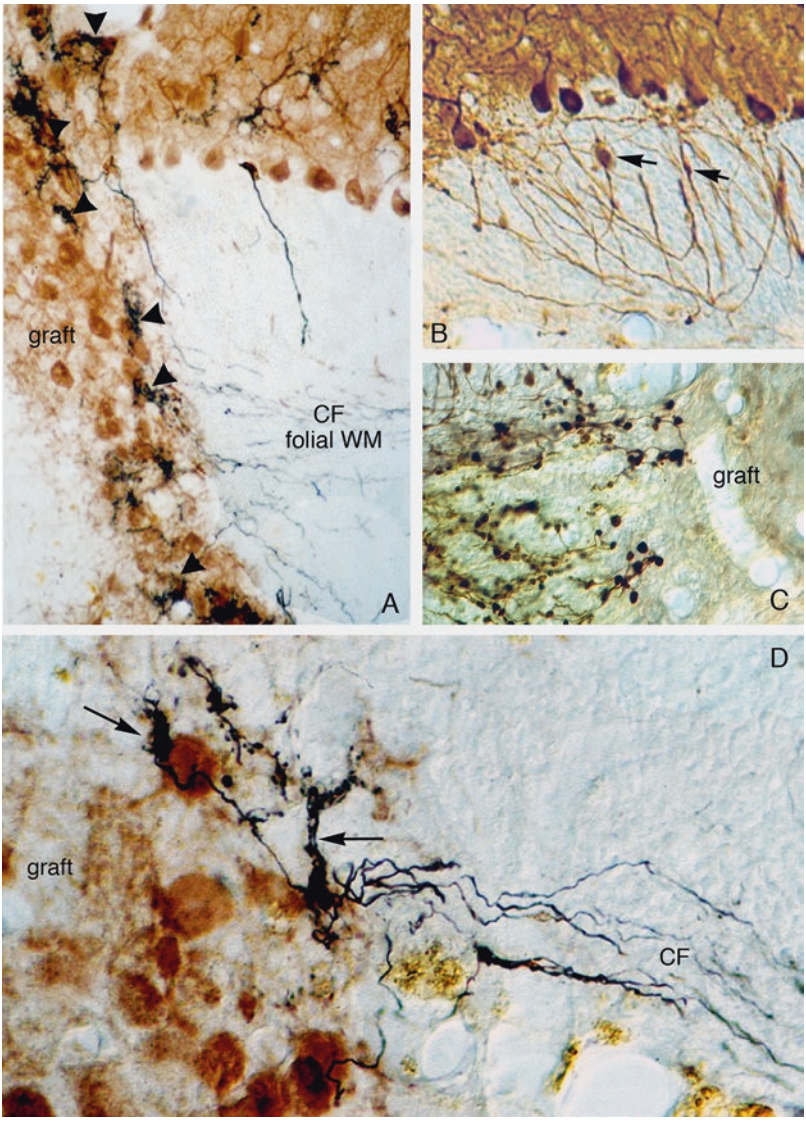

Fig. 5 Axotomy and transplantation, a combined approach in adult rat cerebellum to assess the intrinsic capacity of adult neurons to regenerate in permissive environment. After a cut separating anterior for posterior vermal cortices, the lesion site is filled with rat E13 cerebellar anlage. Survival times were up to 60 days. PCs were analyzed in calbindin-stained sections, and to display climbing fibers, iontophoretic injections of biotinylated dextran amine (BDA) were done in the inferior olive 10 days before fixation. (**a**) Illustrates the narrow band formed by the graft, the entry of the regenerating thin axons, and their peridendritic plexuses around the grafted PC processes. (**b**) Axotomized PCs, with their normal-looking dendritic trees and perikarya, whereas the axons have adopted the arciform shape reported by Cajal. (**c**) Retraction bulbs of axotomized PC axons, ending close to the host/graft interface, have failed their regeneration. (**d**) High magnifications of BDA-filled inferior olivary axons entering the graft and forming small climbing-like terminals outlining the proximal dendrites of the grafted PCs (*arrows*)

In fact, to boost the almost inexistent spontaneous capacity to regenerate of the two cerebellar elements analyzed (PCs and CFs), the combined "lesion and transplant" approach was followed to provide a permissive environment to the cut axons (Fig. 5a) [77]. Several biological materials and survival times were tested with this approach. Segments of aneural peripheral nerve or Schwann cells [82–84], embryonic neocortical tissue [77, 84], and their specific target embryonic cerebellum, first with only 2 months survival [77] and later on up to 12 months to allow for the study of the late sprouting of PC axons [85]. In all cases, the presence of a permissive material allowed CFs to regenerate into the grafts, although with terminal arbors quite different for each type of graft. Only when the graft was embryonic, cerebellum, their specific target domain, the regenerative branches were able to end forming characteristic CFs on the dendritic trees of grafted PCs, as illustrated in Fig. 5a and d of a rat 38 days after axotomy and transplantation. However, at the same survival time after the combined approach, and even in the presence of DCN in the transplant, PC axons neither changed their time of sprouting nor enhanced their capacity to regenerate (Fig. 5c). In conclusion, the use of transplants has been most useful in revealing the differences in the regenerative capacity of two populations of neurons, emphasizing the importance of intrinsic factors in the regenerative process. Grafting has also disclosed that the protracted sprouting of PC axons is not at all equivalent to regenerative capability, corroborating many other studies indicating that the growth in sprouting is regulated by different molecular mechanisms than the growth required for regeneration. Finally, the results also highlighted that cellular changes induced by axotomy in the soma of the injured neuron are the hub for the decision taken of either to start the cell death program or the regeneration program.

The Transplantation of Stem Cells, A New and Fashionable Approach

Origins of the Cells: Embryonic and Adult Multipotent Stem Cells and Immortalized Cell Lines

Although recently, the discovery of the existence of neural stem cells in the CNS of adult mammals has changed our vision of a static adult CNS, it did not however alter the grim reality that is the spontaneous fate of nerve injuries. Indeed, neurologists have known for many years that the loss of a specific population of neurons provokes permanent and irreducible neurological deficits, despite a possible slow, and partial recovery of some of the symptoms. The latter is mostly the result of plastic changes due mainly to collateral sprouting of axons spared by the injury [86, 87] and does not seem to result from spontaneous proliferation of quiescent, local neural stem cells. Despite these negative premises, after the discovery of neural stem cells in the adult brain [23], scientists became more optimistic as they foresaw

the possibility to treat and cure patients with neurodegenerative diseases as well as those with traumatic or ischemic lesions of the brain or spinal cord. Thus, numerous publications appeared on the therapeutic power of exogenous naive stem cell transplantation to repair all types of lesions damaging nervous centers (see in [88]). They used not only adult neural stem cells taken from the central regions known by their abundance in such class of cells (the subventricular zone of the anterior pole of the lateral ventricles or the hippocampus) but also many other classes of multipotent cells of very different origins such as bone marrow-derived mesenchymal stem cells [89] and even immortalized multipotent neural cell lines generated via retrovirus-mediated v-*myc* transfect [90, 91]. Unfortunately, the results of these experiments were rather disappointing because, often, the progeny of the engrafted stem cells either remained undifferentiated [90, 91] or was restricted to glial lineages [92].

Neural Stem Cells from the Postnatal Cerebellum

Concerning the cerebellum, postnatal neural stem cells were found relatively late by Scott Wechsler-Reya's group [93]. These cells had their niches in the cerebellar white matter, and two important features allowed for their identification: (i) the expression of the stem cell marker prominin-1 and (ii) the lack of neuronal and glial cell lineages' markers, even if they could, once transplanted into the cerebellum, differentiate into GABAergic, Pax2-positive interneurons together with astrocytes and oligodendrocytes. These cerebellar stem cells, once isolated from newborn or adult mouse cerebella, could produce clonal neurospheres used for transplantation. It is important to remember that neural stem cells have different potentialities according to the brain region and the age of the donor. Gord Fishell's group [94] has shown, in vivo (transplantation) and in vitro (cell cultures), that the forebrain and cerebellar-derived neurospheres gave their origin to neurons resembling those endogenously found in the brain. In other words, the neural stem cell progenies have regional character and are not totipotent since they have already acquired some differentiation.

The Reprogramming of Somatic Cells into Induced Stem Cells, or Directly into Precise Neuronal Fates, to Provide an Autologous Source of Transplantable Cells

Historical Introduction

John Gurdon's early cloning experiments [95], using somatic nuclear transfer taken from somatic differentiated cells (a tadpole intestinal cell), demonstrated that, when transplanted into an enucleated egg, its reprogramming to a pluripotent stage was possible and gave origin to the complete tadpole. This important discovery

made it possible to put to rest a seemingly inviolable principle of developmental biology: the widespread concept that cell differentiation takes place in one direction only, from pluripotent undifferentiated cells to highly differentiated ones such as neurons. This principle was progressively replaced by the idea that the differentiation process was reversible, thanks to a new mechanism named reprogramming. Reprogramming considers that fully differentiated cells could dedifferentiate and transform somatic cells into pluripotent stem cells through the inductive action of suitable transcription factors and then ready to be differentiated again, but this time in the desired cell class [96]. Shinya Yamanaka's team publication [96], showing that a treatment with only four transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) was enough for this transformation, paved the way to the new stem cell era. Among the numerous and new opportunities offered by this reprogramming process, an essential one is that it can supply a significant and unexpected source of neural stem cells (the induced pluripotent stem cells or iPSCs) for autologous cell therapy, while avoiding the immunological and ethical problems attached with the use of heterologous embryonic stem cells. Furthermore, human-induced pluripotent stem cells (hiPSCs) also allows to build up in vitro models of genetic and acquired cerebellar diseases, providing a superior material to study molecular and cellular pathomechanisms of precise pathways leading to cerebellar degeneration. This, however, as well as the potential of such models for drug screening, is totally out of scope for this chapter.

It soon appeared that it was possible to reduce the number of "Yamanaka factors" from four to three (Sox2, FoxG1, and Brn2 [97]), and even one, as Sox2 in precise conditions was able to induce pluripotent stem cells [98], removing the danger of using the proto-oncogene c-Myc. These derived stem cells were able to be transformed in turn in neural progenitors and neurons. We are living in a time of logarithmic expansion of research on the reprogramming of mature somatic cells into pluripotent iPSCs and from there in unlimited categories of differentiated cells, especially the countless classes of central neurons. Also of great interest is the possibility to reprogram human fibroblast directly into neurons, without passing through the status of multipotent neural stem cells. Indeed, using the combination of three factors (Ascl1, Brn2, and Myt1l), the reprogramming occurred with an induced neuron conversion efficiency of 1.8–7.7 %. These neurons had the membrane properties of real neurons and the capability to establish functional synaptic connection [99].

Regarding the cerebellum, the focus of this chapter, Declercq et al. [100] showed that the Zic3 protein – from the Zic family genes mainly expressed in cerebellar granule cells [101] – was able to maintain the pluripotentiality to the reprogrammed mouse embryonic stem cells. This also minimizes the risk of tumorigenicity that may appear by the inductive action of the "Yamanaka cocktail." This replacement (substitution of c-Myc by Zic3) did not decrease but enhanced the reprogramming efficiency two- to threefold. On the opposite, when Zic3 is blocked by shRNA-mediated knockdown of endogenous Zic3 during iPSC generation, the reprogramming efficiency decreases [100].

Cerebellar Transplantation of Granule Cells

Transplantations of external granular layer's cells have been the starting point in the history of cerebellar grafting [102] and provided the main conditions required for the survival of transplanted cells. Moreover, the granule cell phenotype has also been the most frequently reached after transplantation of whatever class of stem cells in the early postnatal cerebellum [103, 104]. Nevertheless, despite of the existence of excellent murine models of ataxia consecutive to massive death of granule cells, very few studies have been published on the capability of multipotent cells for isotypic neuronal replacement.

An early attempt to replace missing granule cells was carried out by Evan Snyder's group [103], using the vermal anterior lobe of the *meander tail* mutant mouse [105] as a model of agranular cerebellum. The immortalized cell line used for this study (clone C17.2) was generated by retroviral transfection of the proto-oncogene v-Myc in cultures taken from neonatal mouse cerebellum [106]. Injection of these cells on the surface of control newborn cerebella allowed their engraftment, followed by their differentiation program into granule cells. The dendrites of the latter received synapses from host mossy fibers, corroborating their partial synaptic integration [106]. When grafted to the anterior lobe of newborn *mea/mea* mutant cerebella, they survived not only in the granuloprival anterior lobe but also ectopically in the posterior lobe. In the former position, they migrated inward, under the PC layer where they received synaptic contacts from host mossy fibers, as if the grafted neurons acquired a granule cell phenotype and built up a kind of immature inner granular layer. The parallel fibers, the efferent fibers of the grafted cells, were not considered in this work [103].

Naive stem cells of two different origins (cerebellar-derived multipotent astrocytic stem cells and embryonic stem cell-derived neural precursor [107]) have been used in the *weaver* mutant mouse, another model of agranular cerebellum [47]. Neither of them provided neurons with granule cell phenotype. After these negative results, new trials were carried out with human cells taken from either the hindbrain of 5–7-week-old embryos [108] or obtained by stable reprogramming of cultured human fibroblasts, taken from the scalp tissue of patients with traumatic brain injury [109]. The former cells were first propagated in culture with EGF and FGF2 and then oriented toward upper rhombic lip derivatives by treatment with bone morphogenetic proteins (BMPs). When grafted into neonatal rat brain, they generated granule cells that integrated into host cerebellar circuitry. The great advantage of these human multipotent cell lines is their capacity for generation without genetic immortalization. The latter cells, scalp fibroblasts from patients with traumatic brain injury, were directly reprogrammed by the combination of three transcription factors (Ascl1, Sox2, and Oct4), followed by the treatment of three secreted factors (BMP4, Wnt3a, and FGF8b), into human-induced cerebellar granular-like cells (hiGCs) [109]. This protocol is a direct shortcut to convert one adult cell phenotype into a totally different one, without passing through the state of multipotent stem cell. The hiGCs were used to assess their ability to replace missing cells in the cerebellum of $Nmyc^{TRE/TRE}$; tTS, a Nmyc conditional knockout mice [110],

a mutation characterized by a severe microencephaly, particularly with a profound atrophy of about 65 % of the cerebellar mass affecting mainly to granule cells [110]. The cell transplantation provided some positive results, at least regarding motor behavior. However, the morphological study was incomplete and though some markers for granule cells were positive, synaptic integration of the grafted cells was not examined, and the newly originated granule cells did not seem to be equipped with their distinctive "T"-shaped polarity phenotype [109]. It is important to note a major difference between previous trials in *weaver* and *meander tail* mutants and these later experiments. In fact, while the results with the former were obtained after newborn transplantations, those of the third model of agranular cerebellum were obtained with mice 8 weeks old when transplanted. In any case, the problem is again that the experiments providing real evidence of neuronal replacement with synaptic integration were only those done on newborn animals. Therefore, the mice experiments discussed here cannot validate the future use of cell therapy for presumptive clinical use, since valid experiments need to be done with older mice, after cerebellar histogenesis is finished, because in humans the mean age of onset of dominant ataxias is about 30 and 40 years [111], and the plasticity of immature cerebellar tissue is not at all comparable with those of adult cerebellum. It is therefore evident that cell therapy in ataxias with loss of granule cells still remains out of reach.

Cerebellar Transplantation of Purkinje Cells

Due to the difficulties in obtaining PCs from naive stem cells, researchers decided to reprogram in cell cultures the cellular and molecular microenvironments characterizing all the known phases that progenitors should pass through to reach their ultimate identity. They did it either by transfecting with viral vectors, the genes coding for transcription factors involved in their differentiation cascade, or by adding these factors to the culture medium. In such a way, Hideyuki Okano's team [112] induced PCs from mouse embryonic stem cells in a coculture system where the stem cells were floating over a serum-free culture of embryoid body-like aggregates treated with BMP4, Fgf8b, and Wnt3a. Later on a much higher production of PCs was obtained as a result of modifications introduced by Keiko Muguruma et al. [113, 114], who reproduced in cell cultures of mouse embryonic stem cells, the molecular microenvironments containing the inductive signals that native PC progenitors successively receive during their differentiation. As in previous experiments, the stem cells were first oriented toward cerebellar fates by the synergistic addition of insulin and Fgf2 to the culture medium, thus increasing the expression of genes, such as Wnt1, Fgf8, and En2, acting at the midbrain-hindbrain boundary, that regulate the polarity and identity for the specification of the cerebellar plate. However, almost 60 % of the En2-positive cells co-expressed Pax2, a marker of cerebellar GABAergic interneurons and only a few attained the PC fate. The orientation toward the Ptf1a expression [115], and its corollary co-expression of Neph3 in stem cell-derived progenitors necessary for their specification into PCs, was

achieved in a second step, called dorsal specification, by inhibiting sonic hedgehog (Shh) signal transduction with cyclopamine. This inhibition prevented the expression of Atoh1 and the formation of granule cells [116]. Finally, the third and last step, the differentiation of Neph3+/Ptf1a + cells [117] into Corl2-expressing PCs [118] – the earliest specific marker expressed in these neurons – was obtained after cell sorting and purification of the Neph3+ cells kept a few days in co-culture with mouse cerebellar granular cells. This complex treatment allowed for the differentiation of embryonic mouse stem cells into PC progenitors with a very high yield, since over 80 % of the co-cultured Neph3+ cells expressed Corl2.

The Neph3-derived cells obtained from GAD-GFP embryonic stem cells by Muguruma et al. [113] were injected into the subventricular space of the E15.5 cerebellar plates. One month after transplantation, the surviving grafted cells appeared as normally polarized PCs, located at the interphase of the molecular and granular layers and with complete afferent and efferent synaptic integration. Indeed, their axons crossed the granular layer to enter in the white matter axis, and some of them even reached their terminal domains, and established synaptic connections with deep cerebellar nuclear neurons. Therefore, the PCs derived from embryonic stem cells behaved in the same way as PC progenitors transplanted in embryonic cerebellum. Ensuing their normal migratory pathway, they were able to access their orthotopic location and complete synaptic integration (see above and [59]). However, these apparently successful PC replacements remain of difficult clinical application as already discussed for PCs after E12 cerebellar grafts in adult pcd/pcd mutants (see section "The Difficulties to Restore Corticonuclear Projections Argue Against the Possibility of Succeeding Complete PC Replacement by Embryonic Cerebellar Transplants"), and for granule cells (section "Cerebellar Transplantation of Granule Cells"). Indeed, until it can be shown that PCs – derived from iPSCs – can be implanted after the onset of ataxia symptoms, in young adults, not in fetal mice, and that the grafted cells can reproduce the same developmental behavior as in fetuses, this therapy will remain inapplicable to humans.

Prospective Expectations

For many researchers interested in the plasticity of the nervous system, it is obvious that the discovery of the in vivo reprogramming process has awakened the dream of manipulating at our willingness the physiological mechanisms of regeneration available to the brain. This approach of genetic activation of the neurogenesis of adult injured brains, particularly by transformation of reactive astrocyte into newly generated neurons able to replace the missing ones, leaves only –as memory or signal of the injury – a small scar, and does so without transplantation of exogenous biological material. Therefore, regarding its regenerative capability, the nervous tissue could be considered from now on as the others body tissues. Although no one knows what the future holds, these current results give us great hope in a brighter future for reparative neurology.

Note concerning the illustrations: The micrographs illustrating this chapter have been adapted from personal publications in the topic (see references: [29, 46, 55, 56, 58, 60, 62, 64, 77]).

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