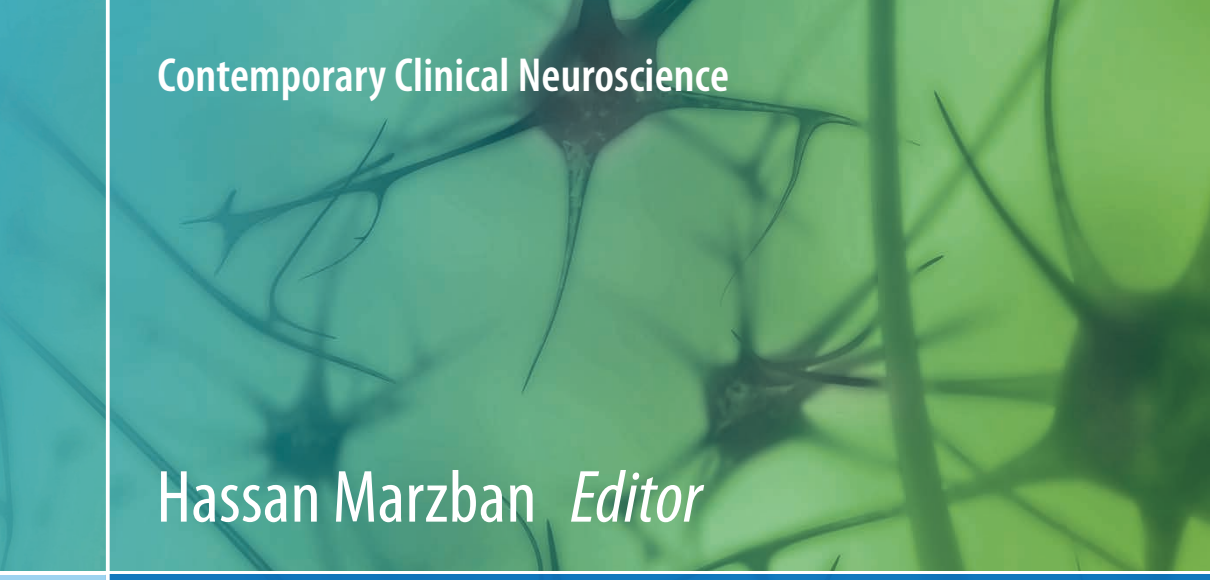

Contemporary Clinical Neuroscience

Hassan Marzban *Editor*

Development of the Cerebellum from Molecular Aspects to Diseases

Second Edition

Springer

Contemporary Clinical Neuroscience

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Editor

Development of the Cerebellum from Molecular Aspects to Diseases

Second Edition

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Preface

This second edition is a revised and updated version of the original book entitled *Development of the Cerebellum from Molecular Aspects to Diseases* published in 2017. The overwhelmingly positive response to the first edition exceeded our expectations and validated the significance as well as the broad reception of our work. In addition to recent advances in cerebellar neurodevelopmental science in health and disease, this encouraged us to provide an updated version, which includes new chapters and discusses the impact of COVID-19 on the cerebellum in related chapters. We believe this work will benefit both basic and clinical scientists in the field.

All updated content and novel chapters were written and carefully reviewed by experts in the field of cerebellar development. Chapters related to the cerebellum and disease, including new additions such as "The Role of Non-coding RNAs in Cerebellar Development," "A Comparative View of Cerebellar Morphology and Diversity in Fishes," "The Role of nNOS/NO on Cerebellar Development in Health and Disease," and "Rehabilitation in Cerebellar Ataxia," extensively cover epidemiology, clinical features, assessment, and management. Furthermore, the effects of COVID-19 on cerebellar pathobiology have been incorporated where relevant.

We greatly appreciate the continuous support and encouragement from peers in the basic and clinical scientific communities, which greatly motivated us to compile a second edition of our initial publication to provide a reference work featuring the most recent developments in cerebellar science.

Winnipeg, MB, Canada — Hassan Marzban

Contents

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, 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.

Keywords Cerebellar nomenclature · Cerebellar histogenesis · Folial pattern · Purkinje cell zones · Cerebellar connections

The Origin of the Cerebellum

The study of cerebellar embryology begins with His' (1888, 1891) 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) [1, 2]. 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

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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).

The general opinion was that the cerebellum originates from the dorsal portion of the first rhombomere [3, 4]. 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 (1989), these cells are present in a broad medial stripe, where labeled and non-labeled Purkinje cells occur together [5]. Labeling is also found of the granule cells. According to Hallonet et al. (1990), the labeled Purkinje cells are found in a V-shaped, rostral region reaching caudally to lobule VIII (Fig. 4) [6]. 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 [7]. Sgaier et al. (2005) and Nieuwenhuys and Puelles (2016) pointed out that the cerebellar anlage rotates from an original rostrocaudal to a mediolateral position, due to the development of the pontine flexure (Fig. 2) [8, 9]. Purkinje cells produced by the ventricular zone maintain their mediolateral 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 [10]. Granule cells produced by the upper rhombic lip do not maintain their original mediolateral position in the adult, due to their lateromedial tangential migration in the external granular layer (EGL).

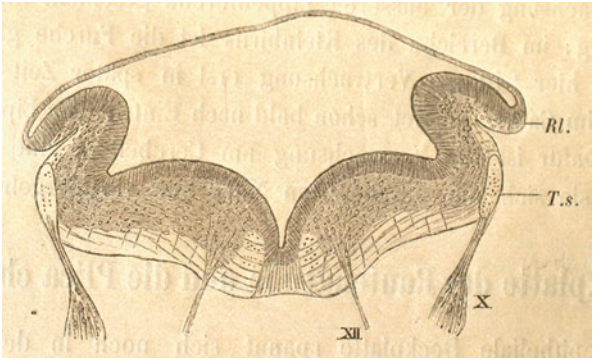

Fig. 1 The rhombic lip of a human embryo at the level of the greatest width of the fourth ventricle (Reproduced from His (1888) [1]). Abbreviation: *R.l.* rhombic lip, *Ts.* solitary tract, *X, XII* vagal and hypoglossal nerves

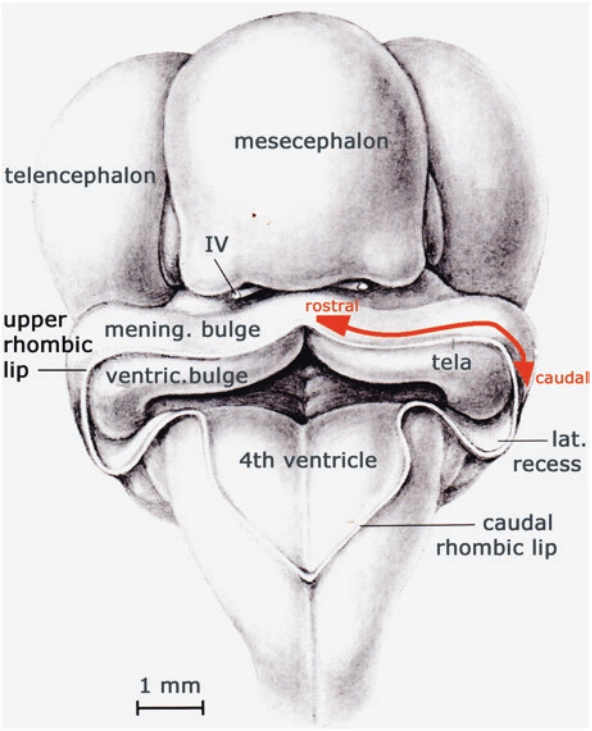

Fig. 2 Cerebellum of a 15-week human embryo (Reproduced from Nieuwenhuys et al. (2008) [123]). Red arrow indicates position of rostrocaudal axis of the cerebellar anlage after the rotation of the cerebellar anlage due to the pontine flexure

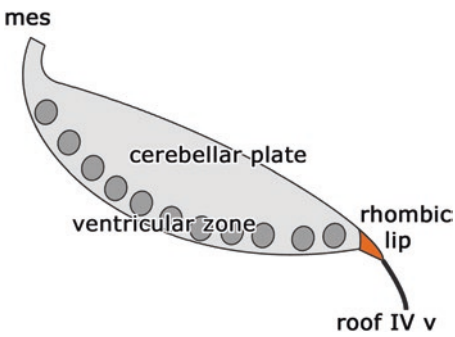

Fig. 3 Diagram of a sagittal section, showing the division of the germinal zone of the early cerebellar plate into the ventricular zone that will give rise to inhibitory neurons and the upper rhombic lip that produces the excitatory neurons of the cerebellum. (Redrawn from Golgowitz and Hamre (1998) [124])

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 (1961) and Pierce (1975) 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; medium and small (presumably inhibitory inter- and nucleo-olivary neurons) between E11 and E17 [11, 12].

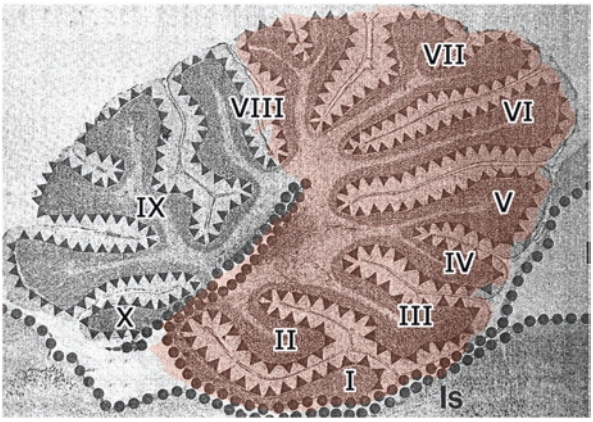

Fig. 4 Sagittal section through a chicken-chimera cerebellum. In the rostral region of the cerebellum, Purkinje cells (triangles) and ependyma (circles) are replaced by quail cells derived from the mesencephalon. (Modified from Martinez and Alvarado-Mallart (1989) [5])

Purkinje cells are born during the same period (E11-13). 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 [11]. These cells give rise to Bergmann glia, astrocytes, oligodendrocytes, and basket and stellate cells of the molecular layer [13, 14]. 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 [15]. 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 [16], for the monkey [17], and for the chick embryo [18]). 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. 2015) and the observation of Englund et al. (2006) that unipolar brush cells are produced by the rhombic lip [19, 20]. Inhibitory neurons of the cerebellum, therefore, are produced by the 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 [21]. In the rat, all Purkinje cells migrate through the more superficially located transitory nuclear layer [22]. 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 (1890a, b, 1909–1911) and his followers (Athias 1897; Lugaro 1894; Popoff 1897) distinguished different phases in the development of the Purkinje cells (Fig. 6a–d) [23–28]. In the first phase of the "disoriented

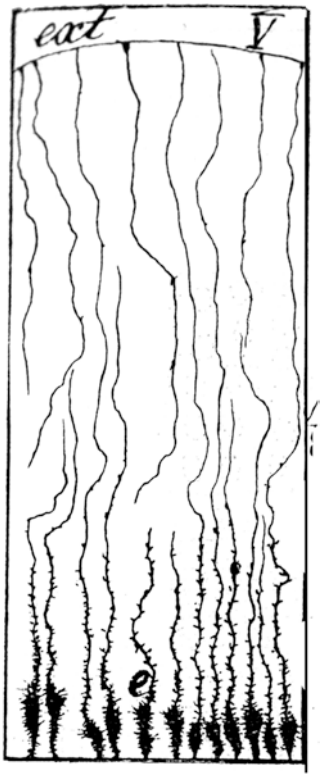

Fig. 5 Neuroepithelial cells (Popoff's spongioblasts) in a 1–1/4 cm. cat embryo. Golgi method (Reproduced from Popoff (1897) [28]. Abbreviations: *e* ventricular zone, *ext* external limiting membrane)

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, II, proximal V and VI, and IX and X and late in distal VI, VII and distal VIII [29, 30].

The development of the climbing fibers closely follows that of the Purkinje cell [23, 25, 28]. Cajal distinguished an early pericellular nest stage where the climbing fiber forms an intracellular 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 (1969) and Morara et al. (2001) [31, 32]. Multiple innervations by climbing fibers of the Purkinje cell was noticed by Cajal and others (Fig. 6e, h). The elimination of redundant climbing

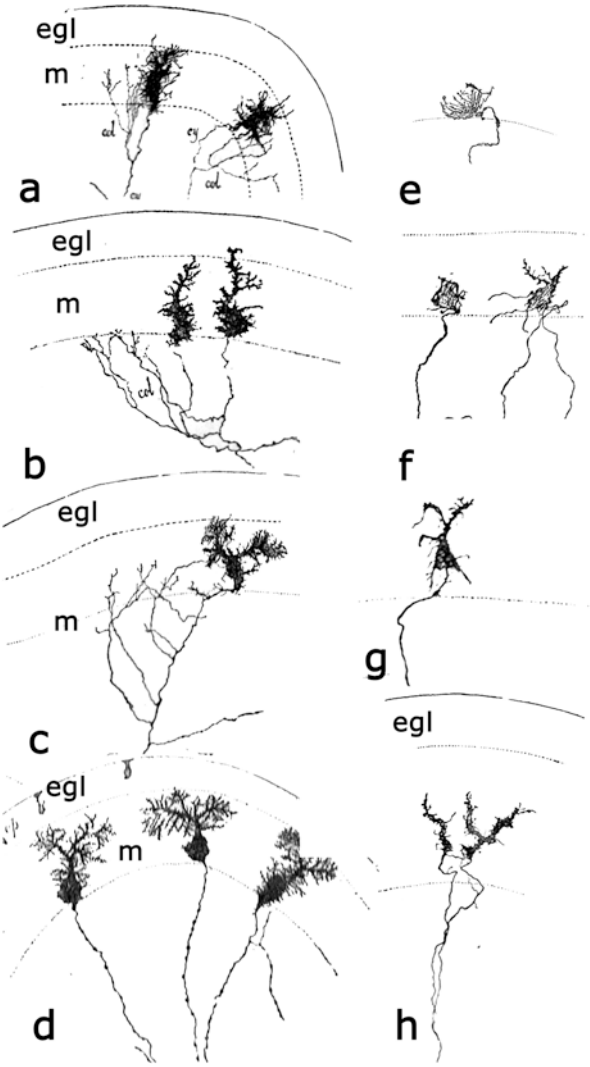

Fig. 6 Stages in the development of Purkinje cells (**a**–**d**) and climbing fibers (**e**–**h**). (Reproduced from Athias (1897) [26])

fibers was shown much later in physiological studies, reviewed by Hashimoto and Kano (2005) [33].

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 (1858), who illustrated it as the stratum granulosum periphericum in the cortex of a neonate dog (Fig. 7) [34]. 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 (1869) distinguished a superficial, tightly packed, and a deep layer with loosely arranged rounded cells in the EGL (Fig. 8) [35]. 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 (1894) in

fish and Herrick (1891) 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 [36, 37]. They observed mitoses in the superficial EGL and identified it as a secondary matrix. Miale and Sidman (1961) 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 third postnatal week [11]. Proliferation in the EGL is regulated by sonic hedgehog, secreted by the subjacent Purkinje cells [38].

In his 1890 paper, Cajal described different cell types in the EGL and the molecular layer (Fig. 9) [23]. 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

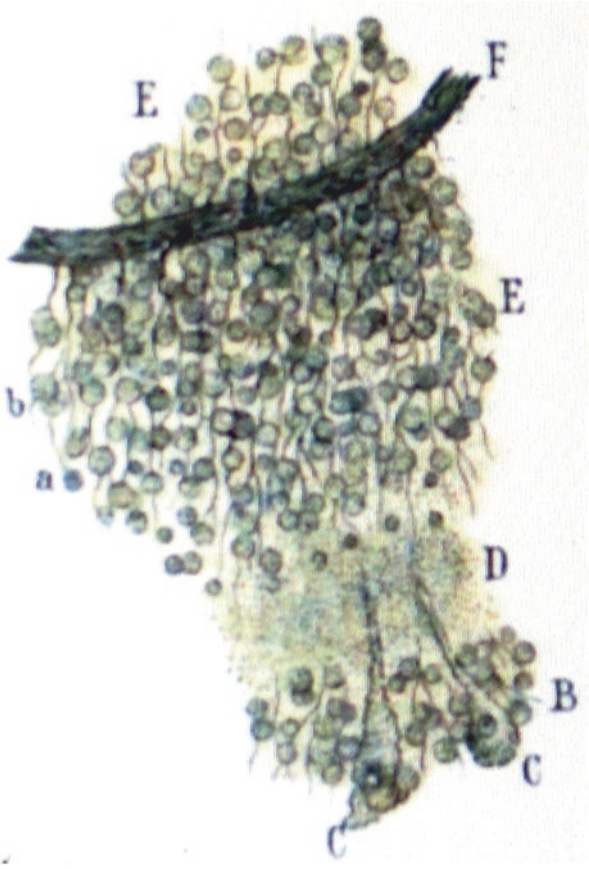

Fig. 7 Cortex of neonate dog showing the stratum granulosum periphericum. Carmin staining (Reproduced from Hess (1858) [34]). Abbreviations: *a* granule cell with processes directed at the periphery, *B* Stratum granulosum centrale, *b* granule cell with filiform processes at both ends, *C* nerve (Purkinje) cells, *D* stratum moleculare, *E* stratum granulosum periphericum, *F* pia mater

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 (1869) [35])

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 upwards. 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 [22].

Granule cell precursors use Bergmann glial fibers for their migration [39]. These fibers, with their typical lateral processes and their attachment to the meningeal surface of the cerebellum, were described by Bergmann (1857) [40]. Bergmann glia

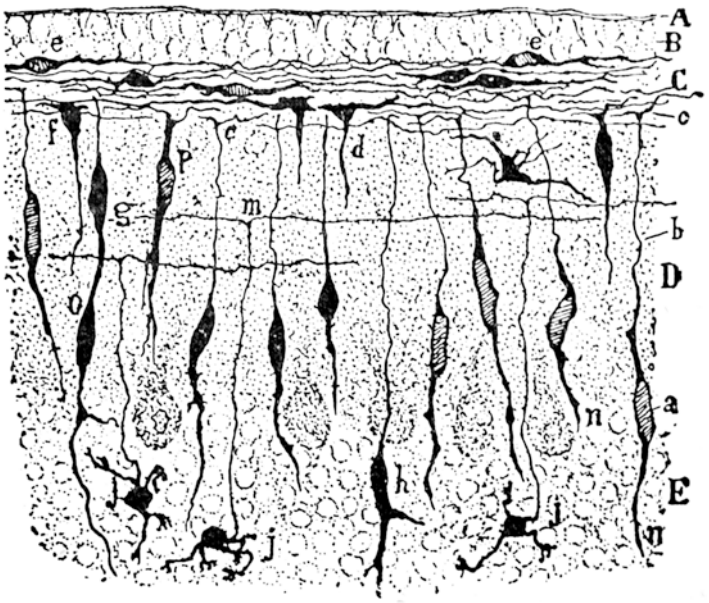

Fig. 9 Neurons in the developing EGL and the molecular layer (Reproduced and relabeled from Cajal (1890) [23]). Abbreviations: *A* cuticula, *a* vertical bipolar cell, *b* ascending process that terminates in c in a bifurcation, *B* layer of epithelial cells, *C* zone of horizontal bipolar cells, *d* 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

has been described as originating from the Golgi epithelial cells by translocation of their cell bodies to the Purkinje cell layer [21], but have also been traced from cells proliferating in the cerebellar white matter [13]. 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 [22, 41]. However, the orientation of the parallel fibers in the long axis of the folia can be uncoupled after perinatal administration of methylazoxymethanol in rats [42].

Development of the Cerebellar Nuclei

The first study of the development of the cerebellar nuclei in different classes of vertebrates is by Rüdeberg (1961) [3]. In the tradition of Bergqvist and Källén (1953), 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 [43]. The dorsal part of the first migration area A gives rise to the

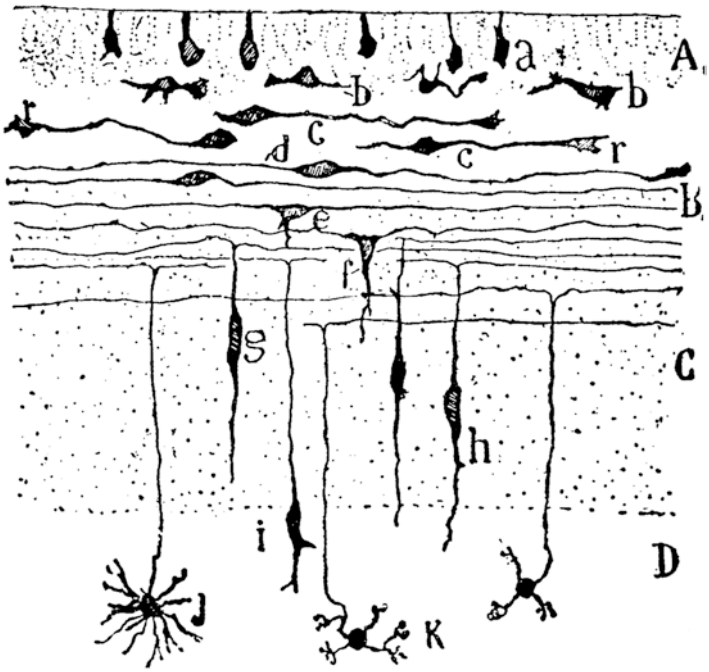

Fig. 10 Stages in the development of the granule cells (Reproduced from Cajal (1909/11) [25]). Abbreviations: *A* external granular layer: matrix, *B* external granular layer: layer of horizontal bipolar cells, *b*,*c*,*d* horizontal bipolar cells, *C* molecular layer, *D* granular layer, *e*,*f* bipolar 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

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; in mammals, it gives rise to the lateral (dentate) nucleus. The interposed and fastigial nucleus stems from ventral parts of migration B. The development of the cerebellar nuclei in cetacea follows the same pattern (Korneliusen and Jansen 1965) [44]. According to Korneliusen (1968), all nuclei in the rat develop from the deep layer of migration B. The nomenclature used by Feirabend (1983) 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 [45]. The two migration layers were also recognized by Altman and Bayer (1985a, b, c) in the rat [10, 16, 46]. 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

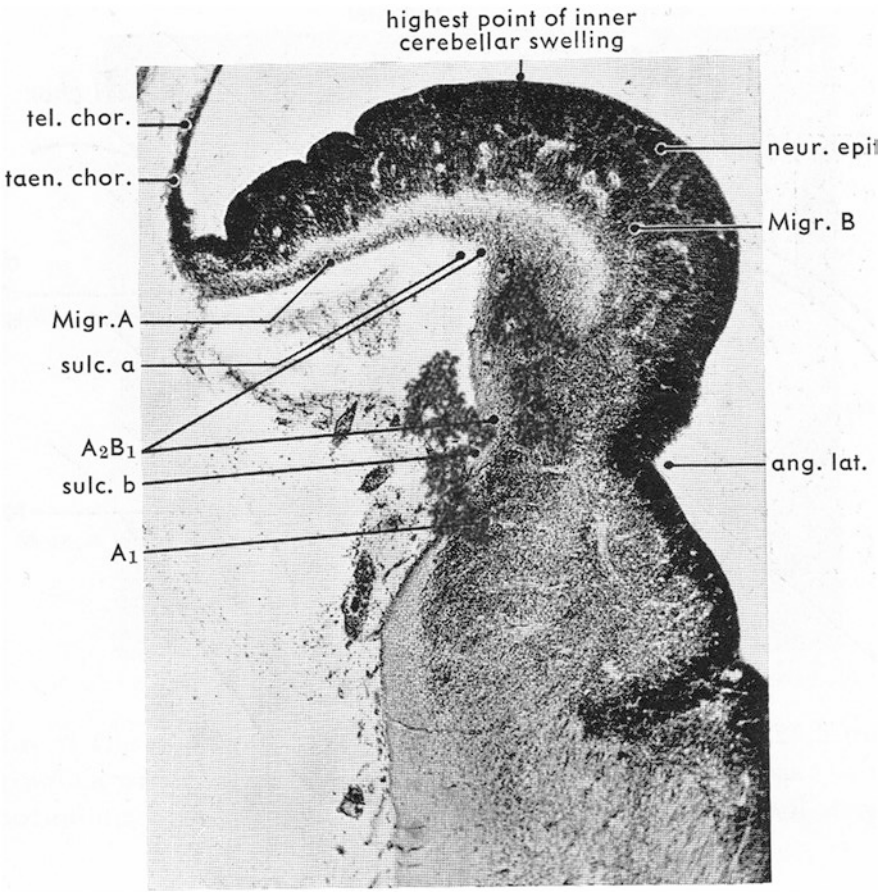

Fig. 11 Transverse section of the cerebellar anlage from a 21 mm human embryo. (Reproduced from Rüdeberg (1961) [3])

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 (1986) (Fig. 12, inset; [47]). The longitudinally oriented neurons will develop into the interposed and lateral (dentate) nuclei. With the demonstration by Machold and Fishell (2005) and Wang and Zoghbi (2005) that glutamatergic neurons of the nuclei are derived from the upper rhombic lip [48, 49], Rüdeberg's migration A, or Altman's nuclear transitory zone became a layer of tangentially migrating neurons destined for the cerebellar nuclei.

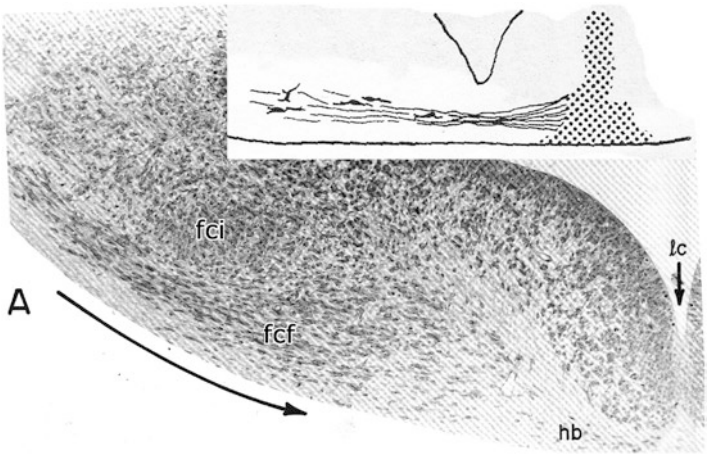

Fig. 12 Transverse section through the cerebellar anlage of a E17 rat embryo, showing the division of the nuclear transitory zone in a group of medially migrating, future fastigial nuclear neurons (fcf) that will give rise to the uncinate tract (hb), and a group of longitudinally oriented neurons (fci), the source of the interposed and lateral nuclei (Reproduced from Altman and Bayer (1985b) [16]). 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 [47]

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 (Voogd 1964, 1969), illustrated in Fig. 13A–C [50, 51]. 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 rostrocaudal 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 (1967) in cetacea [52]. In *Balaenoptera musculus* (blue whale) and *Balaenoptera physalis* (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 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

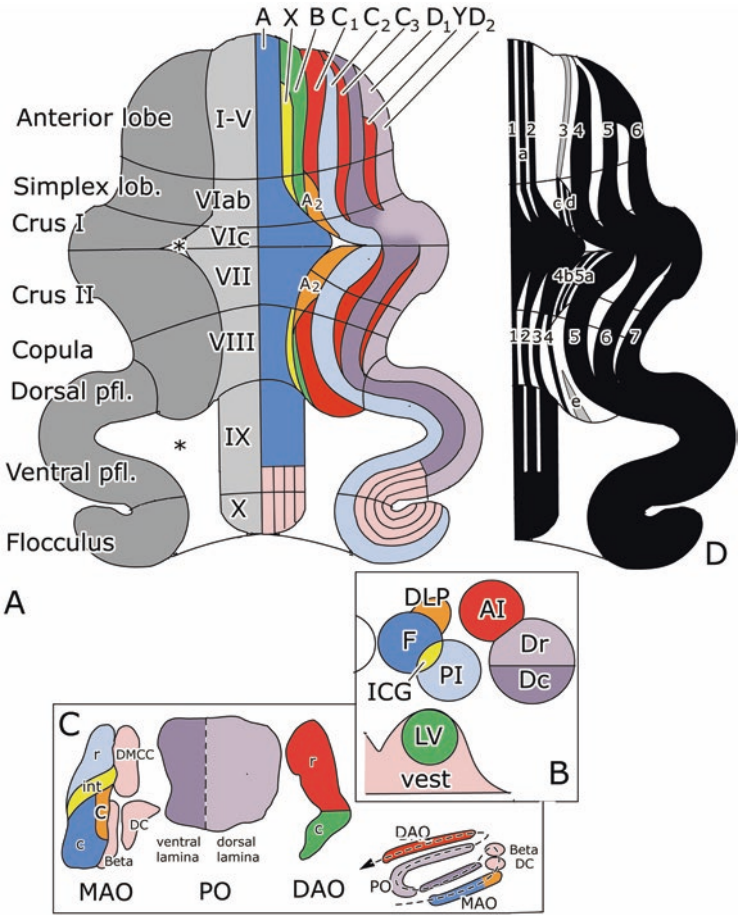

Fig. 13 A. Diagram of the Purkinje cell zones in a flattened map of the cortex of the cerebellum of the rat. The cerebellar and vestibular target nuclei of the zones are indicated in B, the source of their climbing fiber afferents in a flattened map of the inferior olive in C. A map of the distribution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

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 (Korneliusen 1968) (Fig. 14) [53]. 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 (1983) in chick embryos [45]. In

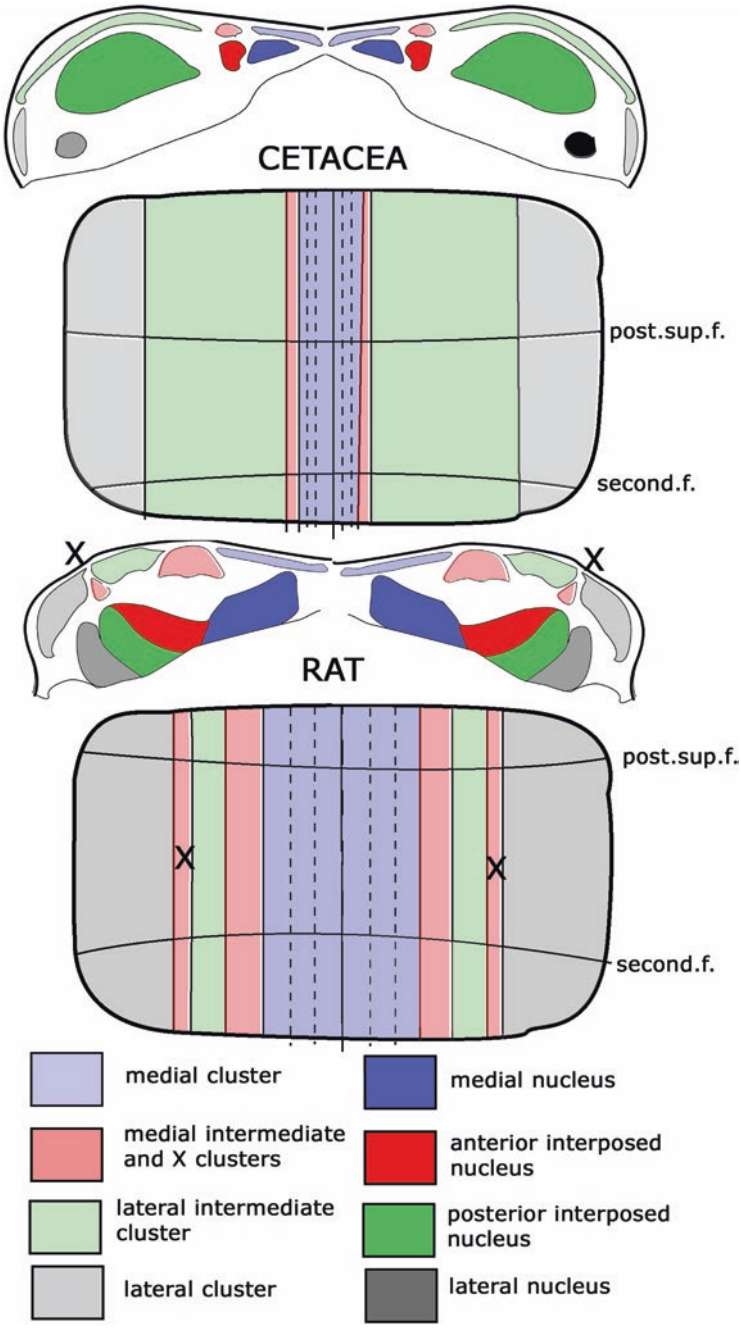

Fig. 14 Transverse section and a diagram of the flattened cerebellar cortex showing Purkinje cell clusters in a 17 cm cr *Balaenoptera physalis* embryo and a 30 mm cr rat embryo. (Modified from Korneliusen (1967, 1968) [52, 53])

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 [54–57]. Cerebellar zonation in early postnatal avian stages was documented by Braun et al. (1986) [58].

Purkinje cell clusters have been identified during the development of the primate cerebellum. The first illustrations in human fetuses can be found in Langelaan (1919) and Hochstetter (1929) [59, 60]. They were studied in macaque monkey fetuses by Kappel (1981) [61]. She distinguished two sets of clusters. Those destined to develop in the adult A, C2 and D1 and D2 zones reach the early, still smooth surface of the cerebellum (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 (1968) paper on the rat corticogenesis [62]. Korneliusen's medial and lateral intermediate and his X zone clearly correspond to the monkey C1, C2, and C3 zones, respectively. In the 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 (1899). The general conclusion of these studies is that Purkinje cell clusters transform directly into the adult pattern of Purkinje cell zones. Nothing is known about the development of the detailed (somato) topical patterns in the Purkinje cell zones [63].

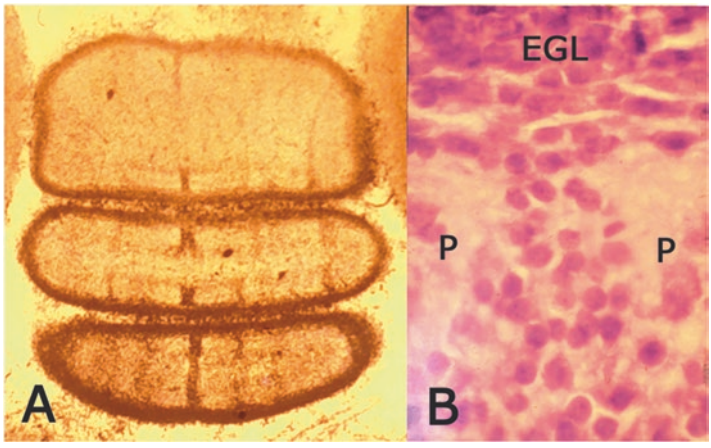

Fig. 15 Granule cell raphes in a 14-day chick embryo. A. Loyez stain of anterior lobe, the EGL, and the granule cell raphes are stained. B. Nissl stain. EGL, external granular layer; P, Purkinje cell clusters. Courtesy Dr. Hans Feirabend

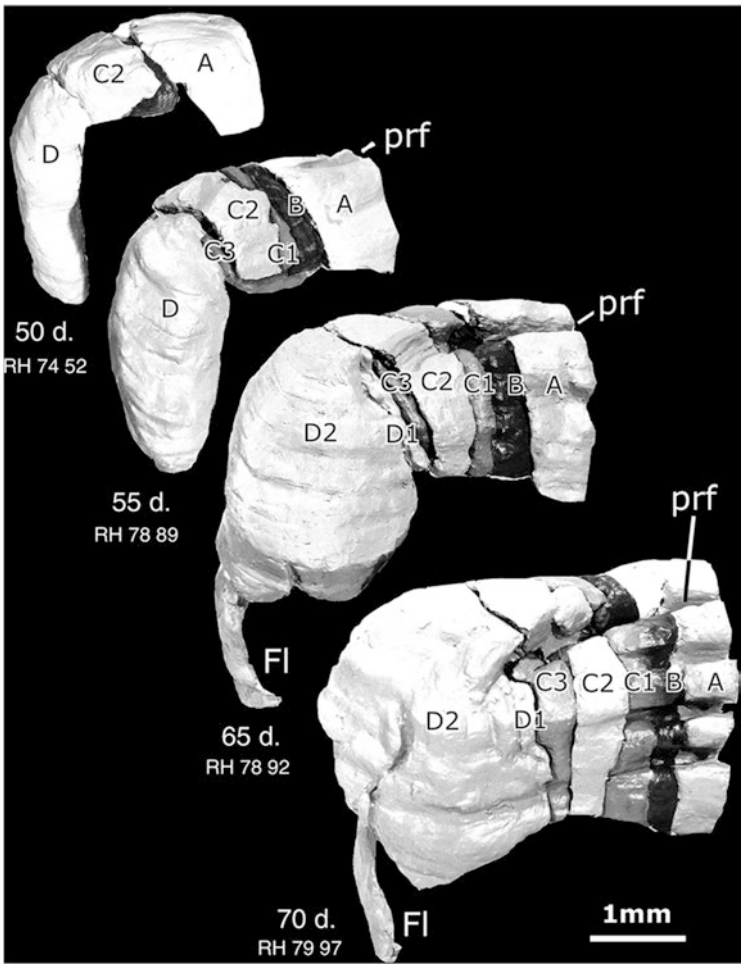

Fig. 16 Photographs of the rostral aspect of reconstructions of the Purkinje cell layer of the cerebellum of four fetuses of the rhesus monkey. Clusters are indicated with different shadings. Note the superficial location of Purkinje cells of the early arriving clusters D, C2, and A in the youngest fetus, 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 (1981) [61])

The role of cadherins, adhesion molecules that play an important role in cerebellar development was reviewed by Redies et al. (2011) [64]. 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 [65, 66]. In mice, these cadherin domains resemble the Purkinje cell zones as they are known in rats.

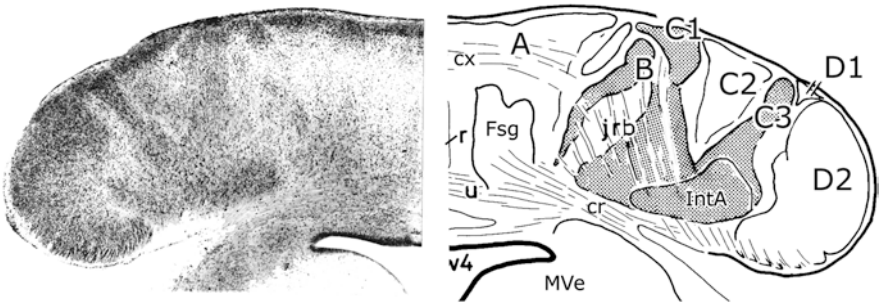

Fig. 17 Coronal section through the cerebellum of a 55-day-old rhesus monkey fetus. Note superficial location of the Purkinje cells of the early arriving clusters A, C2, and D, which still partially cover the later arriving deep clusters B, C_1, and C_3. Abbreviations: *cr* restiform body, *IntA* anterior interposed nucleus, *v4* fourth ventricle. (Reproduced from Kappel (1981) [61])

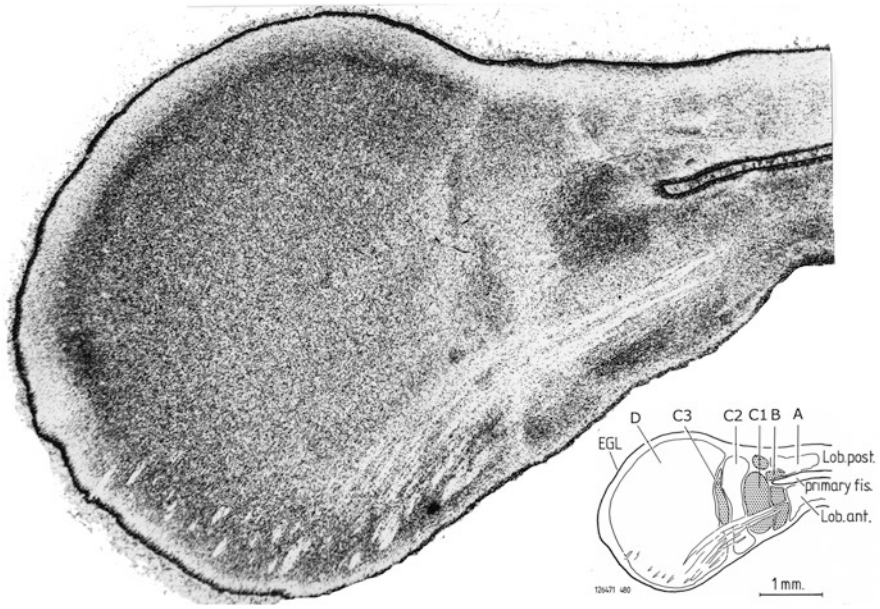

Fig. 18 Purkinje cell clusters A–D in a transverse section of a human 65 mm cr fetus. *EGL* external granular layer

Wassef and Sotelo (1984) and Wassef et. al. (1985) traced the development of Purkinje cell clusters in rats, using markers that are expressed by all adult Purkinje cells [67, 68]. 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

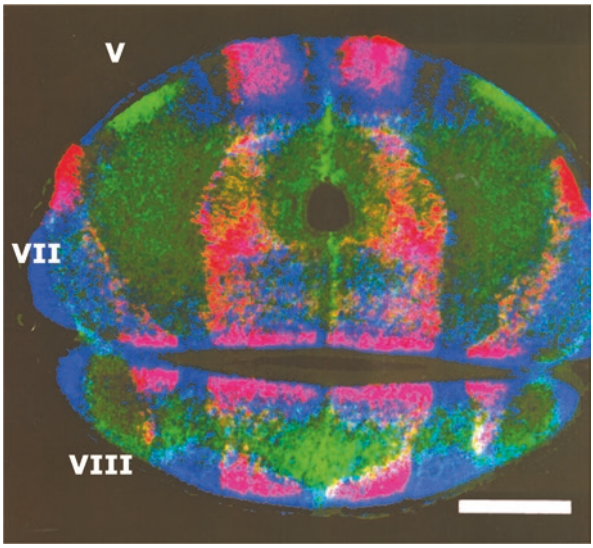

Fig. 19 Zonal distribution of different cadherins in a section through the E11/12 chicken cerebellum. Red, Cad6b, blue, Cad7, green R-cadherin. Scale bar 500 micron. (Reproduced from Arndt et al (1998) [65])

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 (1987: mapQ-113, zebrin I [69]) and Brochu et al. (1990, zebrin II [70]). The epitope of zebrin II was found to be aldolase C [71]. 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 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 [72]. A similar type of development has been found for another late-onset marker for longitudinal zones, heat-shock protein 25 [73]. In studies of the development of the zebrin pattern, bridging the gap between prenatal clusters and adult zebrin-negative and -positive strips proved to be difficult [74].

One of the problems is that zebrin-positive and -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. (2003), Voogd and Ruigrok (2004) and Sugihara and Shinoda (2004) [75–77]. 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 -negative subzones. Earlier publications on differences in birth date between the Purkinje cells of different clusters (Feirabend et al. 1985) were succeeded by the viral-labeling studies of Hashimoto and Mikoshiba (2003) that showed that Purkinje cells in mice born at E11.5 form clusters that will develop into

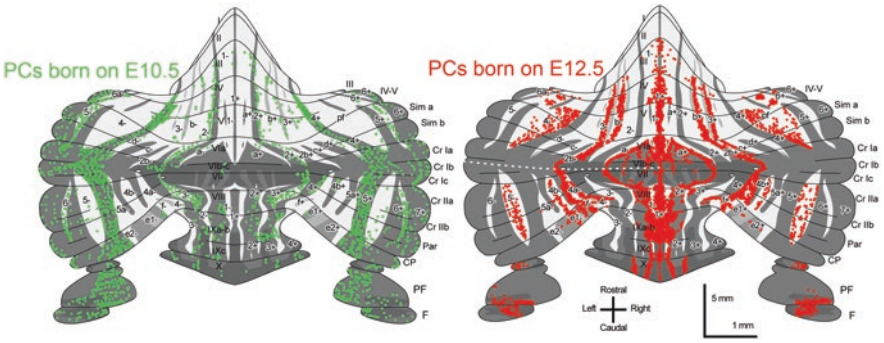

Fig. 20 Distribution of Purkinje cells born on E10.5 and E12.5 superimposed on a map of the zebrin-positive (grey) and -negative strips of the cerebellum of the mouse. Early-born Purkinje cells constitute zebrin-positive bands, late-born Purkinje cells constitute zebrin-negative bands. (Reproduced from Namba et al. (2011) [80])

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 [78–80] (Fig. 20). Earlier, Kappel (1981) found these late-born Purkinje cell clusters to arrive later at the cerebellar surface than the early-born clusters [61]. Just as the number of zebrin-positive and negative stripes increased in recent studies, the number of Purkinje cell clusters identified in E17.5 mice embryos increased to 54 on each side [81]. 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. in Leto et al. (2015) [19].

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 (Flechsig 1876) [82]. The localization of this tract in the restiform body was illustrated by Darkschewitsch and (Sigmund) Freud (1886) [83]. 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 (1898) [84]. The state of the art at the end of the nineteenth century was reviewed by von Bechterew (1899) [85].

According to Tello (1940), 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 [86]. 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 rostrocaudal dimension of the cerebellum (Fig. 23). Tello's observations on the early arrival of primary vestibulocerebellar fibers were confirmed by Morris et al. (1988), using the parvalbumin immunoreactivity of these fibers in rat embryos [87]. 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 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 (2003) [88].

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 (Voogd 1969 [51]) and birds (Vielvoye 1977 [89]). Lakke et al. (1986) traced spinocerebellar axons with WGA HRP in chicken embryos [90]. They enter the rostral cerebellum in Tello's bulbocerebellar fascicle at the seventh incubation day. They course superficially, to enter the cerebellar commissure two 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

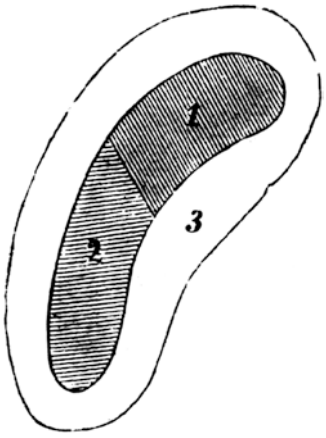

Fig. 21 Diagram of the myelinization of the restiform body in a human fetus. The central, myelinated core consists of cuneocerebellar [1] and dorsal spinocerebellar tract fibers [2]. The unmyelinated periphery consists of olivocerebellar fibers. (From Darkschewitsch and Freud (1886) [83])

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 (1940) [86]). Abbreviations: *n.trig* trigeminal nerve, *n.vest* vestibular nerve

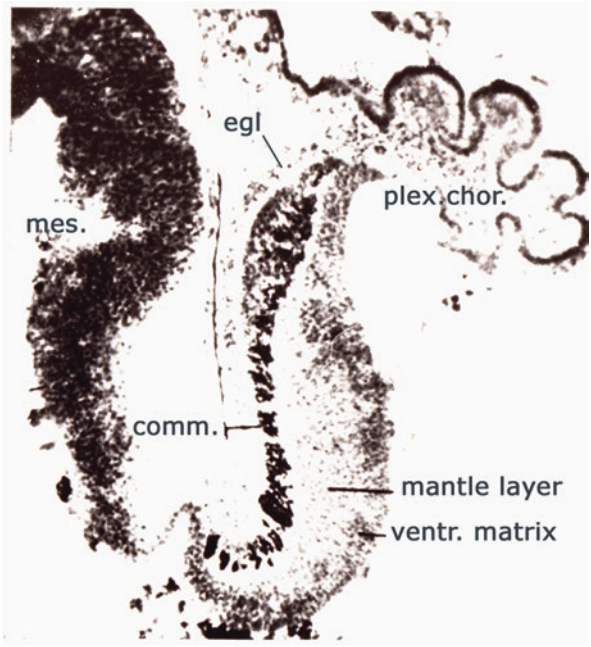

Fig. 23 Sagittal section through a 13 mm mouse embryo; Cajal silver staining, showing the cerebellar commissure (Reproduced from Tello (1940) [86]). Abbreviations: *comm* cerebellar commissure, *egl* external granular layer, *mes* mesencephalon, *plex. chor*. choroid plexus, *ventr. matrix* ventricular matrix

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 [92]. According to Arsénio Nunes and Sotelo (1985), the columnar distribution of spinocerebellar fibers in the rat develops postnatally from a more diffuse stage that was not observed in birds [93]. A distinct topographical relationship of these columns to the zebrin pattern, that is, to the Purkinje cell zones, was described by Ji and Hawkes (1994) [94]. 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 [95].

Little is known about the development of other mossy fiber systems. For the development of the pontocerebellar projection, Bechterew (1885) made an interesting observation [96]. 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 [76]. Tolbert and Panneton (1983) 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

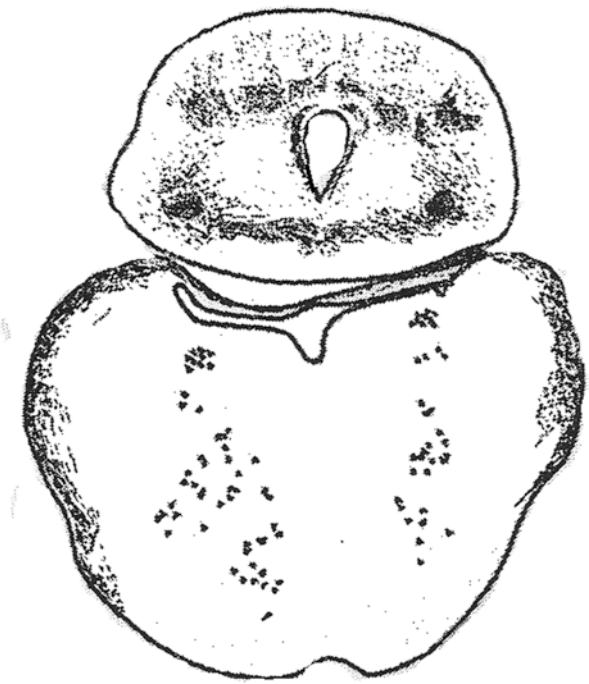

Fig. 24 Bundles of spinocerebellar fibers in an 11-day incubation chick embryo are positionally related to the Purkinje cell clusters. Collaterals are seen to enter these clusters. (Reproduced from Lakke et al. (1986) [90])

dyes [97]. 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 [98, 99]. Nuclear projections are present at P6-8, cortical projections between P8 and 10. After the seventh postnatal week, no cerebrocerebellar projections were present anymore. Earlier, a similar transient pathway from the occipital region of the hemisphere to the paraflocculus was observed in neonatal rabbits [100].

Since the studies of Voogd (1969) and Groenewegen and Voogd (1977), it is known that the topographical organization of the olivocerebellar projection closely matches the longitudinal zonal organization of the corticonuclear projection, their target nuclei and their localization in white matter compartments [51, 101]. 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 E8.5-9 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 (1992), Wassef et al., (1992a, b), and Paradies et al. (1996) (Fig. 26) [102–105]. The cell adhesion molecule BEN was found to be present in

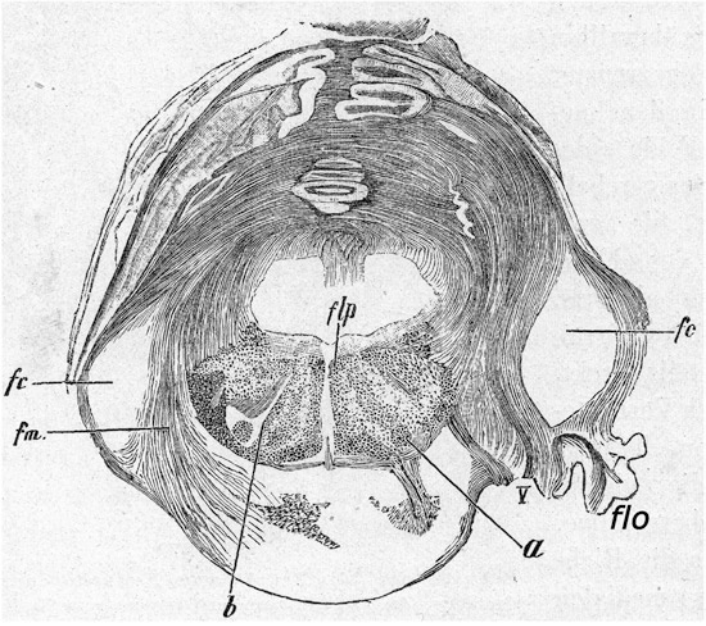

Fig. 25 Transverse myelin-stained section through the brainstem and cerebellum of a human neonate (Reproduced from Bechterew (1885) [96]). Abbreviations: *a* central tegmental tract, *b* superior olive, *fc* cerebral system of the brachium pontis, *flo* flocculus, *flp* medial longitudinal fascicle, *fm* spinal system of the brachium pontis

subdivisions of the inferior olive and Purkinje cell clusters. However, non-BEN-ir clusters also were found to receive BEN-ir olivocerebellar fibers [106, 107]. Irrespective of reversal of the cerebellar plate, olivocerebellar fibers recognize polarity cues in their target region that organize their anteroposterior topography [108]. Ephrins and their receptors are distributed in parasagittal domains in chicken embryos [57]. These domains were found to correspond to the olivocerebellar mapping domains [109].

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 cerebellar nuclei". The development of the brachium conjunctivum was studied in rat fetuses by Cholley et al. (1989) [110]. It emerges from the cerebellar nuclei at E15; at E16 it crosses the

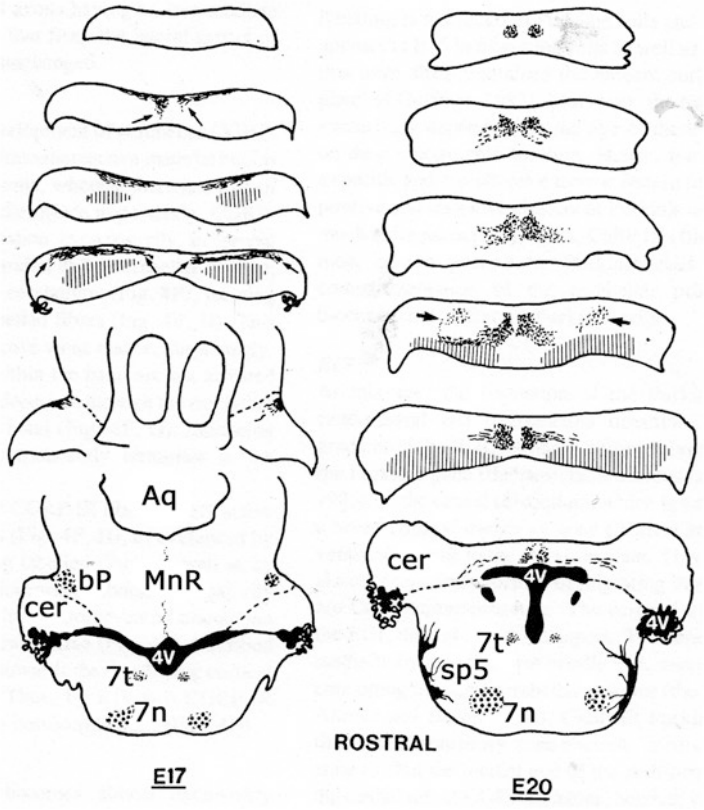

Fig. 26 Location of CGRP immunoreactive olivocerebellar fibers in the cerebellum of a E17 and an E20 rat embryo. CGRP immunoreactive brain stem nuclei and tracts are indicated. Cerebellar nuclei are hatched (Reproduced from Chédotal and Sotelo (1992) [102]). Abbreviations: *4V* fourth ventricle, *7n* facial nucleus, *7t* genu facial nerve, *Aq* aquaduct, *bp* parabrachial nucleus, *cer* cerebellum, *MnR* median raphe, *sp5* spinal tract trigeminal nerve

midline in Wernekinck's decussation [111]. 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 (1895) coined the name sulcus primarius for the first fissure to appear medially in the cerebellum of a 5 cm sheep embryo [112]. Another, unnamed, fissure is present at this stage running along the caudal rim of the cerebellar anlage that 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 (1902) 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" [113]. 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 (1895) in feline and human embryos [114]. 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

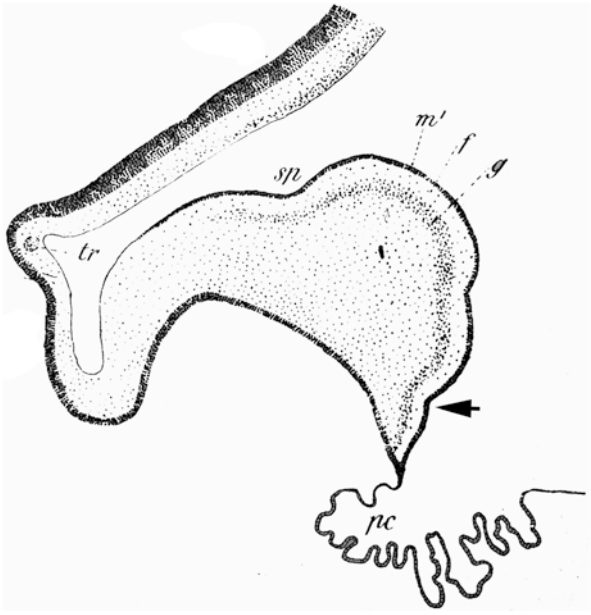

Fig. 27 Sagittal section of a 5 cm sheep embryo, showing the division of the germinal zone of the early cerebellar plate by the primary fissure and an unnamed fissure along the caudal border of the cerebellum (arrow) (Reproduced from Kuithan (1895) [112]). Abbreviations: *f* fibrillar layer, *g* mantel layer, *m'* external granular layer (embryonale Randschicht), *sp* sulcus primarius, *tr* trochlear nerve

to his term paraflocculus, Stroud's names for the primary fissure (the furcal sulcus) and the anso-paramedian lobules have not survived.

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 (1906) (Fig. 28A) [115]. Bolk's (1905, 1906) studies of human embryos [115, 116], that confirmed Bradley's (1903, 1904) earlier observations [117, 118], 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, that is, of parallel fibers,

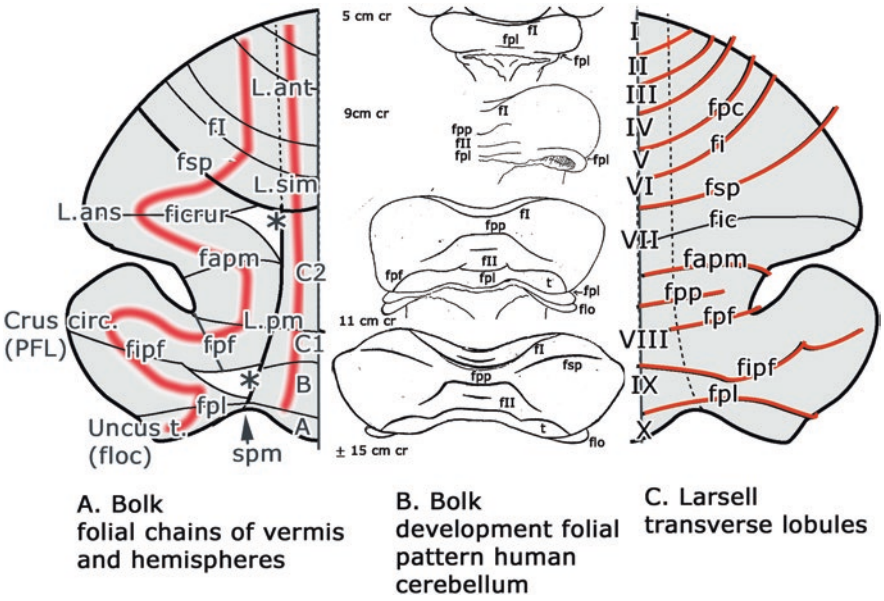

Fig. 28 A. Diagram of Bolk's (1906) Bauplan of the mammalian cerebellum [115]. Folial chains of vermis and hemisphere are aligned in anterior lobe and in the pyramis (C1) and the paramedian lobule, and behave like independent growth centers in the ansiform lobule-lobule C2, and the paraflocculus-flocculus and lobules A (nodule) and B (uvula) segments. B. Drawings of different stages in the development of the human cerebellum. Relabeled from Bolk (1906) [115]. C. Larsell's (1952) transverse subdivision of the mammalian cerebellum [121]. 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)

in these regions [50]. Larsell (1936) 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” [119]. Larsell identified the posterolateral fissure as the first fissure to develop. It separates the primary divisions of the cerebellum, the flocculo-nodular lobe, and the corpus cerebelli, from each other. Larsell subdivided the avian and mammalian cerebellum in ten homologous lobules, indicated with roman numerals [120, 121] (Fig. 28C). The development of the folial pattern in birds was also studied by Saetersdal (1959) [122]. 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, an important structure in the central nervous system (CNS), controls and regulates motor and non-motor functions. It is located beneath the occipital lobe and dorsal to the brainstem. The cerebellum has a well-defined and highly organized structure which folds in lobes and lobules. The cortex of the cerebellum contains different glial cells and eight neuronal cell types and receives inputs from a variety of regions within the CNS and processes the information in a uniform manner. The cerebellar nuclei projects to a variety of different sites within the CNS to regulate motor and non-motor functions. Although much has been discovered regarding the complex architecture of the cerebellum and circuitry, there are significant gaps in our understanding of the broader role of the cerebellum in brain function. This chapter will briefly review the cerebellar embryology and provide an overview of anatomy of the cerebellum.

Keywords Cerebellum · Embryology · Anatomy · Histology · Function

Introduction

Recently, the cerebellum (Latin: "little brain") has drawn the attention of more neuroscientists because not only does the cerebellum involve in motor functions (the regulation of posture, motor coordination, balance, and motor learning) but it also plays a role in non-motor functions such as emotion and cognition. In addition, the

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cerebellum is considered as an outstanding model in the research of neurogenesis and circuit assembly because of its well-organized structure. The cerebellum is a complex organ which makes it difficult to understand its functions and disorders.

First, this chapter briefly reviews the embryological development of this important organ in the posterior cranial fossa. Then, the subdivision of the cerebellum will be elaborated in which the anatomical description subdivides it into lobes, lobules, folia, and zones. There are many different cerebellar subdivisions, as the cerebellum has a unique anatomical organization. On the superior aspect, the cerebellum consists of the 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 rostrocaudal 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 (Fig. 1a, b). Next, the phylogenetic description subdivides the cerebellum into three functional divisions: the vestibulocerebellum, spinocerebellum, and cerebrocerebellum. The cerebellum consists of a uniform layer of cortical gray matter overlying white matter that surrounds four pairs of cerebellar nuclei (CN). The cerebellar cortex consists of three layers from outer to inner: molecular layer, Purkinje cell layer, and granular layer, and CN will be explained in more detail. Finally, connection of the cerebellum to the brainstem via three peduncles (superior, middle, and inferior) and blood supply of the cerebellum will be explained.

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 named neural crest cells [1]. During the third week of embryogenesis, the rostral extent of the neural tube develops into the prosencephalon, mesencephalon, and rhombencephalon. The prosencephalon

Fig. 1 (continued) (**c**) Schematic representation of the cerebellum showing the mossy fibers and climbing fibers convey information to the cerebellar cortex. The mossy fibers synapse on the granule cells and send collaterals to the cerebellar nuclei while the climbing fibers terminate on 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 fibers that contact the Purkinje cell dendrites as well as the basket cells and stellate cells. The Golgi cells receive input from mossy fibers and also project into the molecular layer of the cortex

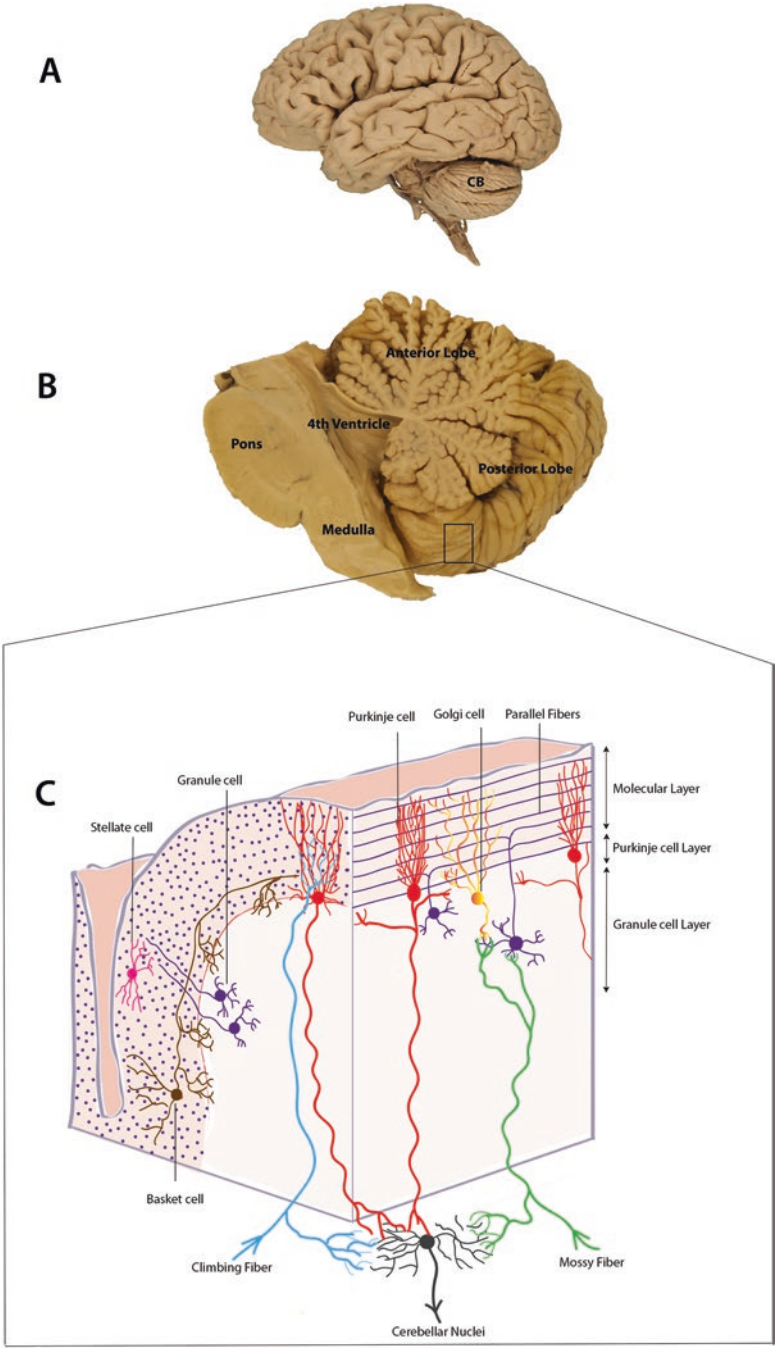

Fig. 1 (**a**) Location of the cerebellum *in situ*. (**b**) Hemisected view of the cerebellum showing the vermis, the locations of the anterior and posterior lobes, and its anatomical relationship to the brainstem.

(continued)

undergoes further development to form the telencephalon and diencephalon. The mesencephalon does not undergo further division while the rhombencephalon is divided 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. This rotation of dorsal r1 results in the conversion of rostrocaudal axis seen in the early neural tube, into the mediolateral axis seen in the mature cerebellum (the wing-like bilateral cerebellar primordia) [2]. As the bilateral cerebellar primordia fuse, the midline vermis is derived from the rostromedial ends while the cerebellar hemispheres are derived from the more caudolateral components of the rhombencephalon [3].

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 includes the Purkinje cells, candelabrum cells, Golgi cells, Lugaro cells, stellate cells, and basket cells. All of these neurons produce gamma-aminobutyric acid (GABA) neurotransmitter and reside in the two outer layers of the cerebellar cortex, Lugaro cells locate between the molecular layer and the granular layer, and Golgi cells within the granular layer. The neurons derived from the rhombic lip produce glutamate neurotransmitter, which includes the large excitatory neurons of the CN (projecting to the diencephalon and brainstem), unipolar brush cells and granule cells (the most numerous cells in the brain) [4, 5].

Anatomy and Histology of the Human Cerebellum

Gross Anatomy and Internal Structure of the Cerebellum

Cerebellum in the posterior cranial fossa, anteriorly separated from the pons and the medulla by the fourth ventricle, and superiorly separated from the occipital lobe by the Tentorium Cerebelli (Fig. 1a, b). Anatomically, cerebellum consists of two large bilateral hemispheres which are merged together by a median part called vermis. Morphologic and phylogenetic descriptions subdivide cerebellum differently.

Cerebellum consists of outer grey matter (cerebellar cortex) and inner grey matter (CN) which is embedded in white matter. The white matter consists of afferent and efferent fibers has branched to form a tree-like appearance, so-called the arbor vitae (tree of life). Afferent and efferent fibers pass through three cerebellar

peduncles that emerge in the hilum of the cerebellum. All the aforementioned structures have been elaborated under following titles [6–8].

Subdivisions of the Cerebellum

The morphological description subdivides the cerebellum into lobes, lobules, folia, and zones. Fissures divide the cerebellum into three lobes in the rostrocaudal 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 large posterior lobe from 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. Several anatomical fissures divide the cerebellar lobes into 10 smaller lobules (lobule I to X) in which enumeration is based on the Schmahmann classification. Lobular subdivisions of cerebellum are described in Table 1 [9, 10]. The lobules undergo further in folding and leads to the formation of "folia," which are particularly prominent in human cerebellum. It seems that folia of human cerebellum are not uniform in size and shape [11]. 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. It is noteworthy that some folia have their own white matter (core of the folium). Cerebellar folium has crown (apex) and walls (lateral surfaces) separating from the neighbor folia by fissures (interfolial) called "fundi" [11]. Zones are distinct: (i) medial or vermal zone, (ii) intermediate or paravermal zones on either side of vermal zone, and (iii) hemispherical zones on either side lateral to the paravermal zones.

As mentioned before, the phylogenetic description subdivides the cerebellum into three functional divisions: the vestibulocerebellum (archicerebellum), spinocerebellum (paleocerebellum), and pontocerebellum or cerebrocerebellum

Table 1 Lobular subdivisions of cerebellum. Enumeration is per the Schmahmann classification

Lobes	Subdivisions of vermis	Lateral extensions in hemispheres
Anterior lobe	I (Lingula)	Frenulum
	II–III (Central lobule)	Ala
	IV–V (Culmen)	Quadrangular lobule
Posterior lobe	VI (Declive)	Lobule Simplex
	VIIA (Folium)	Superior semilunar lobule (Crus I)
	VIIB (Tuber)	Inferior semilunar lobule (Crus II)
	VIII (Pyramid)	Biventral lobule
	IX (Uvula)	Tonsil
Flocculonodular lobe	X (Nodule)	Flocculus

(neocerebellum) [12–16]. This subdivision is based on connections to other brain sites and their respective roles in regulating movement and other non-motor functions.

Vestibulocerebellum is the oldest component of the cerebellum which includes the flocculonodular lobe. The cortex of this lobe receives input via mossy fibers from the vestibular ganglia on the ipsilateral side as well as input from the vestibular nuclei of the brainstem. 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 regions outside of the cerebellum. Briefly, the Purkinje cells of the cortex send inhibitory projections to the fastigial nucleus as well as the ipsilateral vestibular nuclei of the brainstem. The fastigial nucleus, which serves as the principal cerebellar nucleus of the vestibulocerebellum, sends excitatory bilateral projections to the vestibular nuclei through the inferior cerebellar peduncle. These projections play an important role in coordinating the vestibular ocular reflex to control eye movement. The vestibular nuclei also send descending fibers within the vestibulospinal tract which play a critical role in maintaining balance through activation of the antigravity 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. Therefore, the vestibulocerebellum participates in the control of eye movements and maintains posture and balance.

Spinocerebellum is the second functional component of the cerebellum which consists of the midline vermis and a narrow portion of cortex on either side of the vermis referred to as the paravermis. This component is referred to as the spinocerebellum which is bulk of the input provided by ascending tracts in the spinal cord. The spinocerebellum receives some major inputs: (i) the dorsal spinocerebellar tract that transmits proprioceptive, cutaneous, and pressure information from the lower trunk and lower extremity (on the ipsilateral side); (ii) input from the cuneocerebellar tract, which carries somatosensory information from the upper trunk and upper extremity; (iii) input from the ventral spinocerebellar tract that transmits information regarding the activity of circuits within the spinal cord involved in regulating motor activity; and (iv) inputs from a number of brainstem nuclei including the reticular formation. The Purkinje cell axons of the paravermis project to the interposed nuclei which in turn project to both cerebral cortex and the brainstem are involved in regulating the limb musculature and the activity of the spinal cord motor neurons projecting to the upper and lower limbs, respectively. The vermis project to fastigial nuclei are primarily involved in regulating axial musculature. Thus, the spinocerebellum participates in regulating both axial and limbs musculature to control balance, posture, and locomotion.

Pontocerebellum (cerebrocerebellum or neocerebellum) is the largest and phylogenetically newest component of the cerebellum which consists of the large hemispheres immediately lateral to the spinocerebellum. The pontocerebellar cortex receives input principally from the contralateral cerebral cortex, particularly frontal

and parietal lobes, via the pontine nuclei forming cortico-ponto-cerebellar pathways. The axons of Purkinje cells in the pontocerebellar cortex project to the dentate nuclei. Some fibers of pontocerebellum project to the cerebral cortex (premotor and primary motor cortices) via ventrolateral thalamus, and the descending neurons from the cerebral cortex form a large component of the lateral motor system. Some other fibers project to the inferior olivary nucleus of the medulla, via the red nucleus which projects back to the pontocerebellum and dentate nucleus forming a feedback loop to the cerebellum. The neocerebellum is particularly well developed in higher mammals and play a critical role in coordinating the muscle activation required for performing fine motor skills of the distal extremities (particularly upper limb), planning of motor activity, and cognitive functions.

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 [14, 15].

The Molecular Layer This layer contains of stellate cells and basket cells but is dominated by the dendrites and axons of other neurons. It receives input from neurons of the inferior olivary nucleus of the medulla, and these fibers are referred to as climbing fibers. The climbing fibers make abundant excitatory synaptic connections with the proximal dendritic tree of Purkinje cells (Fig. 1c) [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 fibers that run parallel to the cortical surface and make synaptic connections with the dendritic tree of numerous Purkinje cells (Fig. 1c). The stellate cells of the molecular layer are inhibitory interneurons that produce GABA neurotransmitter and these cells are located primarily in the outer part of the molecular layer. These cells also receive input from parallel fibers and make synaptic contacts with the dendritic tree of Purkinje cells. Finally, the basket cells of the molecular layer are also GABAergic neurons and are located in the inner portion of the molecular layer. Basket cells receive excitatory input from the parallel fibers 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 This layer consists of the large cell bodies of the Purkinje cells and candelabrum cells [17]. Purkinje cells send an extensive dendritic tree into the molecular layer. The dendritic tree of a single Purkinje cell receives excitatory inputs from a single climbing fiber of the inferior olivary nucleus and numerous inputs from parallel fibers of the granule cells. The Purkinje cell is of particular importance because it represents the sole output of the cerebellar cortex. They are GABAergic and projects almost solely to the CN [18]. The exception to this rule is

the Purkinje cells of the vestibulocerebellum that also project to the vestibular nuclei of the brain stem. 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 This layer is the innermost layer of the cortex and consists of granule cells, Golgi cells, unipolar brush cells, and Lugaro cells. The granule cells, developed from upper rhombic lip, are the most abundant neurons (99% of cerebellar neurons) in the human nervous system and are packed tightly within the granule cell layer [4]. They receive excitatory input from mossy fibers, which are the principal input into the cerebellum (Fig. 1c). Mossy fibers originate from numerous sites within the nervous system, including pontine nuclei, nuclei of the reticular formation, vestibular nuclei, and the fibers of the spinocerebellar tracts of the spinal cord. The granule cells, which produce glutamate neurotransmitter, extend their axons into the molecular layer where they bifurcate into the aforementioned parallel fibers 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 fibers 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. These are also GABA-ergic neurons that extend their dendrites into the molecular layer where they receive synaptic input from the parallel fibers 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 cells (Fig. 1c). Unipolar brush cells are neurons within the superficial part of the granule cell layer, and like granule cells are glutamatergic neurons. These cells are more abundant in the vestibulocerebellum than other parts of the cerebellum, and are closely associated with mossy fibers 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.

The cerebellar cortex also receives projections from a variety of areas of the brain including the locus coeruleus (noradrenergic fibers), raphe nuclei (serotonergic fibers), mesencephalic tegmentum (dopaminergic fibers), and the hypothalamus (histaminergic fibers) [16]. 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 afferents' projection to the cerebellum is not well understood. Within the cerebellar cortex, the connections and links between the parallel fibers of granule cells and the dendrites of inhibitory cells such as Purkinje cells, and also

connections between the mossy fibers, Purkinje cells, and other neurons, makes 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 (fastigial, interposed (consists of emboliform and globose nuclei), and dentate nuclei) that receive input from the cerebellar cortex as well as the collaterals of all fibers projecting to the cerebellar cortex [16]. The first generated cerebellar neurons 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 fibers and climbing fibers 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 This nucleus is the smallest and most medial CN. The neurons of this nucleus receive input from the Purkinje cells of the vestibulocerebellum. (i.e., flocculonodular lobe). In addition, it receives input from Purkinje cells of the vermis that receive input from the vestibular ganglion directly or indirectly via the vestibular nuclei. The neurons of the fastigial nucleus project to the vestibular and reticular nuclei in the brainstem. As mentioned previously, some of the Purkinje cells of the flocculonodular lobe also send direct (inhibitory) projections to vestibular nuclei of brainstem.

The Interposed Nucleus It is located lateral to the fastigial nuclei in the paravermis, and composed of the globose nucleus (located medially) and the emboliform nucleus (located laterally), 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 receive input from the cuneate nucleus (via the cuneocerebellar tract), 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 premotor cortices of the frontal lobe.

The Dentate Nucleus It 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 fibers and mossy fibers projecting to the lateral hemispheres that have their origin in the inferior olivary 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 cerebellar peduncles, respectively [16].

The Superior Cerebellar Peduncle It consists primarily of efferent fibers 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 afferent contained within this peduncle are primarily fibers of the ventral spinocerebellar tract that project as mossy fibers to the granular layer of the spinocerebellum and send collateral branches to the interposed nuclei.

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

The Inferior Cerebellar Peduncle It contains of fibers 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 fibers within the juxtarestiform body form the mossy fibers projecting to the granular layer of the vestibulocerebellum. The efferent fibers 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 fibers that project from the brainstem and spinal cord to widespread areas of the cerebellum. This includes fibers of the dorsal spinocerebellar tract and cuneocerebellar tract projecting to the spinocerebellar cortex as mossy fibers with collateral projections to the interposed nuclei. In addition, fibers originating from the inferior olivary nucleus projecting to the molecular layer of the cerebellar cortex known as climbing fibers (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 fiber of the inferior olivary nucleus projects to a few Purkinje cells, while each Purkinje cell makes synaptic connections with only one climbing fiber.

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) [15, 16]. These arteries are derived from the vertebral-basilar arterial system that supplies the posterior circulation of the brain.

Posterior Inferior Cerebellar Artery (PICA) 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 fibers of the inferior cerebellar peduncle.

Anterior Inferior Cerebellar Artery (AICA) 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.

Superior Cerebellar Artery (SCA) The SCA branches off 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.

Conflicts of Interest The authors confirm there is no conflict of interest.

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Cellular and Genetic Programs Underlying Cerebellum Development

Andrew K. Lawton, Ryan Willett, and Alexandra L. Joyner

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 two progenitor zones: a ventricular progenitor zone producing astrocytes and all inhibitory neurons, and 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. We describe key transcription factors that regulate development of the two progenitor populations and the lineage relationships of the neurons and astrocytes produced by each. We conclude with a discussion of cerebellar foliation that compartmentalizes these cell types into the final three-dimensional working structure.

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

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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 rhombomere 1 (r1, Fig. 1a, b). Remarkably, the volume of the human cerebellum increases ~10x between 20 and 40 weeks of gestation, with the surface area increasing much more due to the formation of folia and lobules [4–6]. The mouse cerebellum undergoes maximum growth and foliation after birth (Fig. 1a–d). Given the late development of the cerebellum compared to other brain regions, the cerebellum is particularly sensitive to environmental and clinical factors that impact on growth (or cause injury) around birth [7]. A better understanding of the factors that regulate progenitor cell expansion, production of

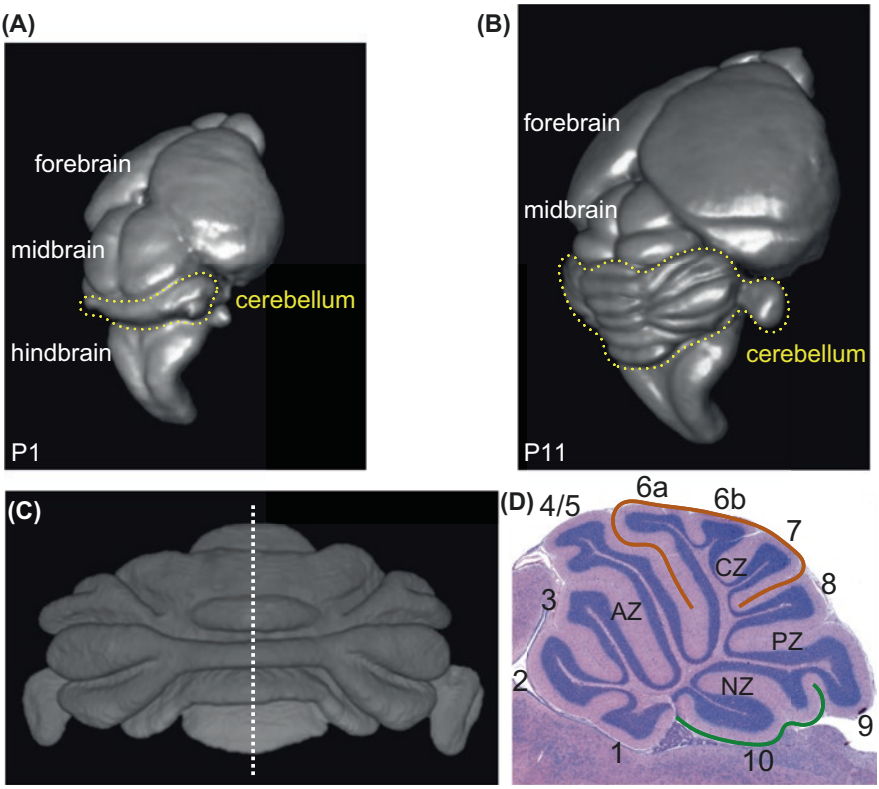

Fig. 1 The cerebellum forms in the dorsal anterior hindbrain and has its major growth and foliation after birth. (**a–c**) micro-MRI images illustrating mouse postnatal cerebellum development (outlined in yellow) (based on [88]), and (**c**) highlighting distinct foliation patterns in the medial vermis and lateral hemispheres [123]. (**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)

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. Towards that end, several recent works profiling the transcriptome of cells from mouse and human cerebella in a variety of conditions and ages provide a wealth of regulatory networks and their complex interactions that initiate and develop the cerebellum [8–12].

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 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) [13]. Interestingly, most of the interneurons and astroglia are generated from intermediate progenitors that leave the VZ and proliferate after birth in the cerebellar cortex [14–17] (Fig. 2b, c). The second

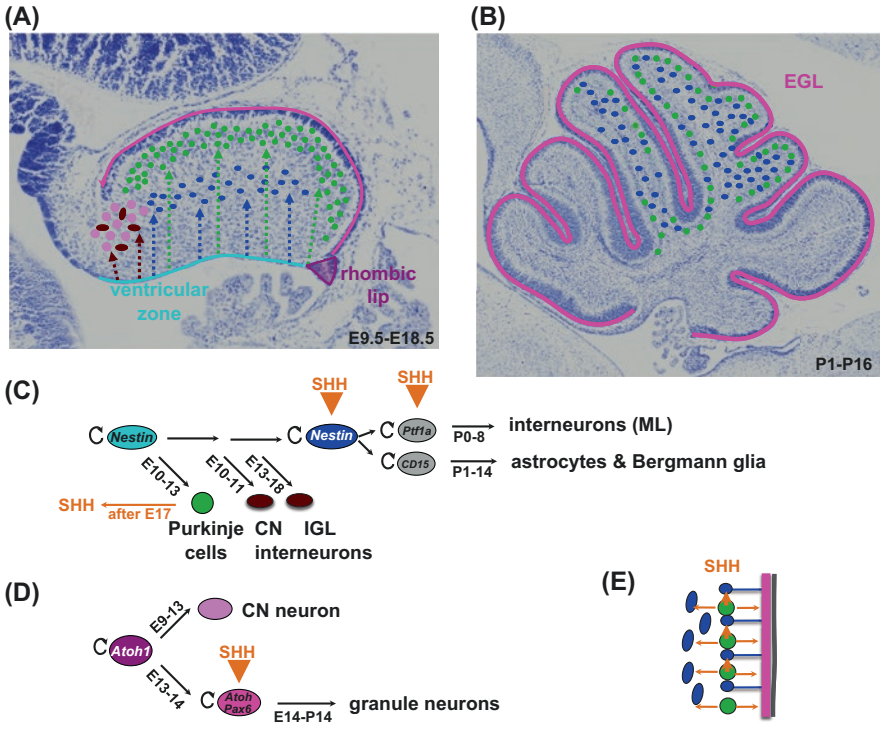

Fig. 2 Two progenitor zones produce all the neurons and the astroglia of the cerebellum at particular time points. (**a**) Midline Eosin-stained sagittal section of E13.5 cerebellum with ventricular zone (turquoise) and rhombic lip (pink) indicated and the cells that arise from the zones color coded as in **c** and **d**. (**b**) Midline sagittal section of E18.5 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**

cerebellar progenitor zone is called the rhombic lip (RL), and generates the excitatory neurons of the cerebellum, primarily the granule cells and projection neurons of the cerebellar nuclei (CN) [18–20] (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 is housed in the external granule cell layer (EGL) that covers the surface of the cerebellum during development and generates granule cells that migrate inwards 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 within the cerebellum appears to be the VZ outside the cerebellum, likely, the midbrain and/or ventral r1 [21–23].

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 [24] (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 [25]. 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

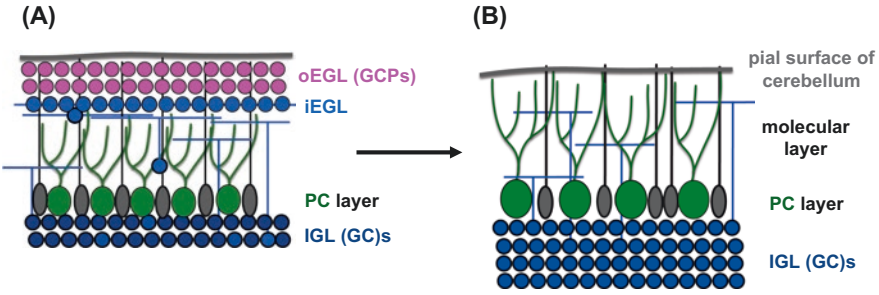

Fig. 3 Schematic drawing showing granule cell development. (**a**) During development (E15.5-P14), the cerebellum is covered with granule cells organized in a layer called the external granule cell layer (EGL) which is divided into an outer layer (oEGL) of dividing progenitors (GCPs) and inner layer (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). 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. (**b**) After P14 the EGL is exhausted with all GCs now present within the IGL. This leaves the molecular layer, and its numerous synaptic connections, at the surface of the cerebellum

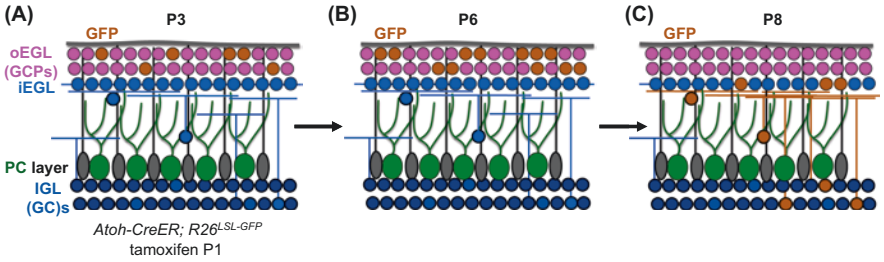

Fig. 4 Schematic illustrating genetic inducible fate mapping (GIFM). An *Atoh1-CreER* transgene is expressed only in granule cell precursors (pink, GCPs) in the outer external granule cell layer (oEGL). A reporter allele, $R26^{LSL\text{-}Gfp}$, expresses GFP in cells that have active Cre. Tamoxifen 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

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 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 r1 around embryonic day 9 (E9) in mouse [26–29]. 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 [30–32]). 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 [33], is sufficient to induce and pattern the midbrain and r1 [34, 35], and is necessary up until E12 for cerebellum development [33, 36]. The secreted factor WNT1 is also expressed near the isthmus and is required for development of the midbrain and cerebellum [37, 38]. The molecular interactions of FGF8 with the transcription factor OTX2, required in the midbrain, and GBX2, required in the hindbrain, have been reviewed extensively, and we refer you to a detailed review by Martinez [31]. 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 [39–41]. The engrailed homeobox transcription factors (EN1/2) are key patterning genes regulated by 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 are involved in regulating growth and foliation of the cerebellum [42, 43]. 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 [44–46].

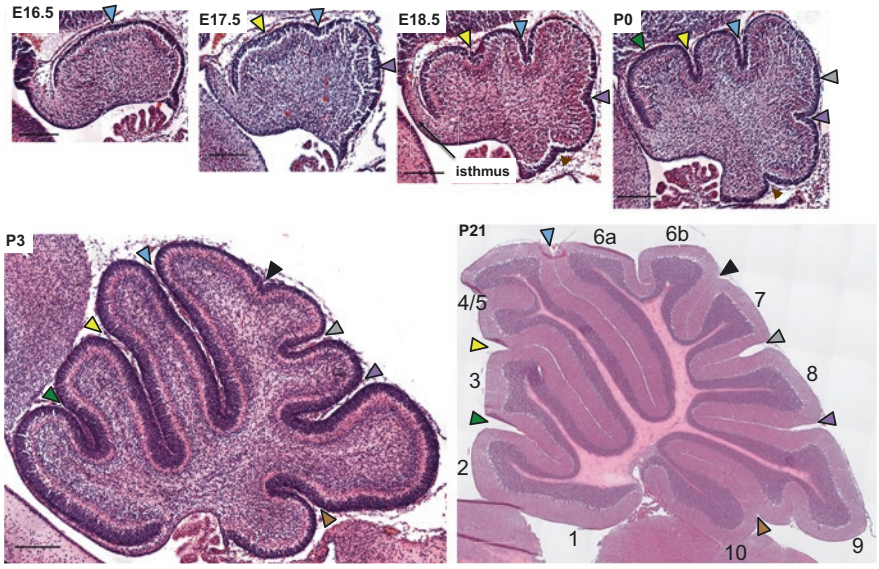

Fig. 5 Stereotypical formation of fissures during mouse cerebellum development. Midsagittal H&E sections of the cerebellum at the indicated stages. The same fissures are indicated by colored arrowheads. The lobules are numbers at P21. Line indicates 200 μm for E18.5-P3 and 500 μm for P28

Ventricular Zone Lineage

Cumulative genetic fate mapping using a line of mice in which *Cre* was inserted by gene targeting into the *Ptf1a* gene (knock-in) (*Ptf1a*^{Cre}), demonstrated that only inhibitory and not excitatory neurons are generated from the VZ [13] (Fig. 2c). Traditional ^{3}H thymidine or BrdU birth-dating experiments and GIFM using *Ascl1*^{CreER} revealed that Purkinje cells and interneurons of the CN are the first neurons to be born during E10–13 [1, 15] (Fig. 2d). Other interneurons are then born in an inside (IGL) to outside (outer molecular layer) spatial progression from an intermediate Nestin-expressing stem/progenitor (NEP) that resides in the white matter of the lobules [14, 15, 17, 47, 48]. 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 [40, 49]. 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 [22]. 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 [23]. A recent fate mapping study argues mouse oligodendrocytes are derived from the hindbrain [21]. Curiously, a small population of Bergmann glia is born at around E13.5 [15], but most are born after birth during the major growth phase of the cerebellar cortex [14, 15, 47, 50]. 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 anterior-posterior oriented stripes [15, 51]. Purkinje cells initially settle into an aggregate of cells called the Purkinje plate at E14.5 before migrating outwards 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) [15, 52]. 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 [53]. While Purkinje cells are born embryonically, a subset seems to maintain an immature status and can undergo cell division in response to ablation of many Purkinje cells to replace the lost cells [54]. However, this plasticity is only observed immediately after birth and is greatly diminished by P5.

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) [55]. 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 a ramified

espaliered fashion within the sagittal plane (P8 onwards, [56]). 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 CN can be seen as early as E15.5 in mice [55], and electrophysiological recordings can be made early postnatally. While the vast majority of Purkinje cell axons project into the CN, Purkinje cells of the flocculus, paraflocculus, and the nodulus of the vermis (lobule 10) instead route into the vestibular nucleus of the hindbrain. The activity and arborization of Purkinje cells exhibit regional and developmental differences with nodular Purkinje cells showing simpler dendritic branching [57].

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 [14–17]. Elegant genetic fate mapping studies combined with marker analysis were used to address the location and lineage relationships of stem/progenitors in the neonatal cerebellar cortex [14, 50]. Using several *CreER* lines (*GLI1*^{CreER}, *Tnc*^{CreER}, *Ptf1a*^{CreER} knockin alleles) to mark NEPs 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 [14]. 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. Another study addressed the location of the multipotent and unipotent progenitors using a *Glast*^{CreER} allele [50]. 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 in the IGL, whereas progenitors in the white matter generate astrocytes in the white matter and interneurons based on a clonal analysis. A more recent study showed that the white matter and Purkinje cell layer NEPs that produce astroglia express *Hopx*, whereas a separate *Ascl1*-expressing progenitor generates interneurons [47]. At P0 the *Hopx*-expressing progenitor generates some interneurons as well as astroglia, but by P5 is mostly dedicated to producing astroglia.

Nestin-expressing progenitors situated along the inner edge of the EGL have been proposed to produce GCPs [58], but it seems possible they normally give rise

to interneurons in the white matter. Interestingly, the plasticity of NEP fates is enhanced after injury, at least for *Hopx*-expressing NEPs in the Purkinje cell layer which respond to irradiation or genetic-induced loss of granule cell progenitors at P1. The cells undergo adaptive reprogramming by expanding in number, then expressing *Ascl1* which seems to allow them to switch their fate from glial to neuronal, and then they migrate to the EGL to repopulate the depleted pool [47, 59–61].

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 interneurons, astrocytes, granule cells, and oligodendrocytes [14, 62]. 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 [63]. 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), [16, 64, 65] which signals to both GCPs and NEPs [14] (Fig. 2c–e). SHH signaling in GCPs is required for their proliferation and viability after E16 [64, 66, 67]. Furthermore, deletion of *Shh* in Purkinje cells or ablation of HH signaling in NEPs reduces expansion of the pool of Tnc^{CreER}-labeled white matter stem/progenitor cells and production of interneurons and astroglia [14]. In addition, application of SHH to cerebellar slice cultures stimulates interneuron production [68]. Purkinje cells can coordinate growth of all cell types produced in the cerebellar cortex except, possibly oligodendrocytes, via SHH secretion (reviewed in [69]). 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 when it is mutated all cerebellar inhibitory neurons are lost and astrocytes are depleted [13, 70, 71]. Some VZ-derived mutant cells are transformed into RL-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 [13, 72]. The related bHLH protein-encoding gene *Ascl1* plays a more limited role in generation of cerebellar interneurons [15, 47]. 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 [73]. Genes that regulate Bergmann glia generation and function are absolutely critical for cerebellar growth,

foliation, and formation of a normal cortical architecture, likely because they play both structural and signaling roles [74].

Rhombic Lip Lineage

The 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 three cell populations: post mitotic CN, proliferating GCPs, and unipolar brush cells. Lineage tracing and birth-dating studies have shown that the earliest population of cells emerging during E9.5–E12.5 accumulate in two clusters of cells bilaterally symmetrically displaced from the midline, known as the nuclear transitory zone [19, 20]. Immature excitatory CN cells migrating from the RL 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 [75]. Excitatory CN neurons play a critical role in the proper balancing of the cerebellar circuitry. Reduction in the number of excitatory CN cells by mutation of *En1* and *En2* or expression of diphtheria toxin results in loss of their synaptic partners, the Purkinje cells. This in turn reduces the number of granule cells, interneurons, and astroglia that are produced likely because less SHH is present in the cerebellum [76, 77].

Cells leaving the RL from E13.5 onwards become cerebellar GCPs [19]. These cells remain at the cerebellar surface for the duration of embryonic development and form a dense proliferative layer called the 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 three paired nuclei in mouse: (from medial to lateral) the fastigial, interpositus, and dentate nuclei. The three individual nuclei are clearly distinct by birth in mouse, and TBR1 or BRN2 expression generally mark the fastigial nuclei or the interpositus and dentate nuclei, respectively [75]. The dentate nucleus of the human CN is greatly expanded compared to mouse, likely related to the vast expansion of hemisphere lobules. This expanded nucleus also has a unique transcriptome-based cell type compositional balance compared to the other nuclei which share more similarities with mouse [78]. Additionally, two separate nuclei are found in human in the place of the mouse interpositus: the human globose and eboliform 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 mouse Purkinje cells by E18.5 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 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 CN projection neurons [20, 79]. One function of ATOH1 is to induce *Gli2* expression, and thus to enhance SHH signaling in GCPs [80], and likely regulate many other genes required for granule cells proliferation (e.g., *MycN* and cyclin D1) and differentiation [81]. There appears to be an antagonistic relationship between the RL protein, ATOH1 and the VZ transcription factor PTF1a, as mis-expression of either protein in the complementary progenitor zone leads to inhibition of the other gene [82]. Mossy fiber neurons also require *Atoh1* for their development [20].

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 [83] has 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 GCPs primarily undergo symmetrical divisions to expand the number of the cells in a clone during postnatal development [84–86]. 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 E17.5 produces an average of 250 granule cells, requiring at least eight cell divisions.

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 an orientation bias of GCP cell division in the anterior-posterior axis along with tangential migration within the EGL [85]. Conversely, as GCPs differentiate into nascent GCs and descend into the IGL, they favor a medial-lateral spread within the growing lobule. 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 [53, 86] (Figs. 3 and 4). Curiously, the cell bodies of new GCs settle at random depths in the underlying IGL. Prior to migrating to the IGL, granule cells are motile and explore a variety of shapes as they move and exchange neighbors within the EGL [87]. The base of each fissure that separates the cerebellum into lobules acts as a boundary to the movement of GCPs [85]. 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 [53]. Thus a picture emerges that the central zone has a general developmental delay that continues for days after cessation of development in the rest of the cerebellum. There are apparent medial-lateral differences in granule cells as well. Granule cells from the hemispheres are more susceptible to raised levels of SHH signaling as in models of SHH-driven medulloblastomas tumors preferentially form in the hemispheres and not the vermis [88]. A full picture of the regional dynamics of growth in the vermis and the hemispheres will be illuminating.

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 [89–93]. By birth, these axons defasciculate and innervate the developing Purkinje cell multilayer, with each Purkinje cell receiving multiple climbing fiber inputs [94]. The supernumerary climbing fibers 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 [95–97]. Mossy fibers arrive in the cerebellar anlage between E13.5 and E15.5 [98] 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 [99].

Development of Cerebellar Foliation and Relationship to Afferent and Efferent Circuitry

During development, the cerebellum increases in size and undergoes dramatic progressive folding transforming the smooth outer surface into a highly foliated collection of lobules separated by fissures (Figs. 1 and 5) [100]. 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 and the synapses at the outer 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 [101]. 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 ([101, 102]. There is also a spatial relationship between Purkinje cells and the CN 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 [103]. The pattern of foliation varies depending on the medial-lateral position in the cerebellum (Fig. 1c), and fissures form in a specific temporal sequence (Fig. 5). In mouse, as in all mammals, the medial cerebellum, or vermis, has 10 primary lobules created by folds that are all aligned in the anterior-posterior axis [101, 102]. 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). This thickening and indentation creates an out-of-phase profile where the lowest point on the surface is also the thickest. At this time, local Bergmann glia direct their fibers to the center of the indention (Fig. 6b) [104]. 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 [104, 105].

As discussed previously, the proliferation of GCPs in the EGL and the resulting growth of the cerebellum is dependent on SHH supplied by the underlying Purkinje cells [64, 66, 67]. 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. When the level of SHH is increased beyond wild-type levels, the mouse cerebellum is larger and has an extra fissure [67]. 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 mutations that activate HH signaling in the GCP-lineage results in the SHH subgroup of medulloblastoma [83, 88, 106, 107].

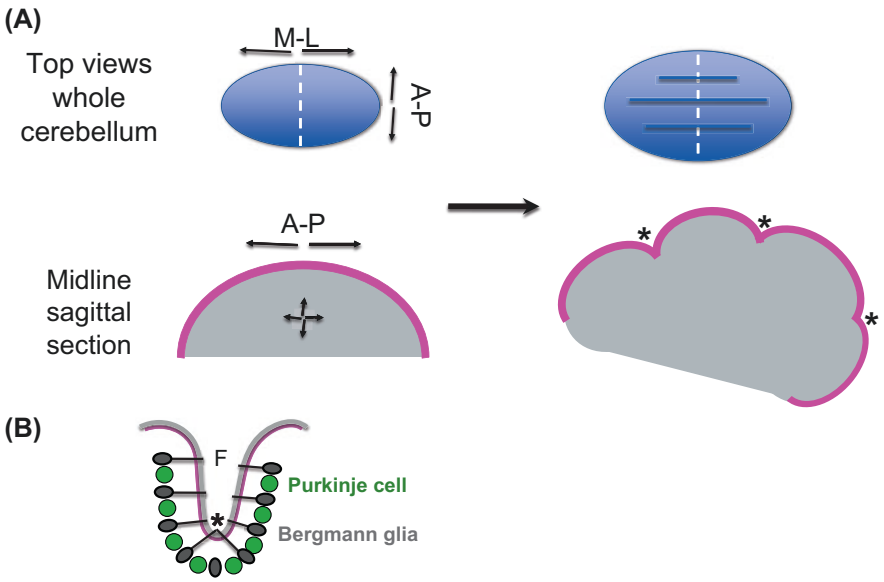

Fig. 6 Model of cerebellum foliation based on differential expansion of layers. (**a**) As the outer layer, the EGL (pink) expands more rapidly than the inner mass of the cortex (grey), the EGL buckles creating anchoring centers (*). White dotted lines in top views indicate where sagittal sections are 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 the anchoring centers as the cerebellum continues to expand. *A-P* anterior-posterior, *M-L* medial-lateral

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 are more similar to the central zone [53]. The lobules are effectively "closed" as granule cells do not disperse across fissure boundaries. This isolation allows any lobule-specific granule cell behavior to fine-tune the shape of the lobules [85].

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 cerebellum is covered by the pial surface as well as the endfeet 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 [74, 108, 109].

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 of, 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 [110]. 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 are 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 [42, 45]. 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 [104].

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 [111–116]. Like the cerebral cortex, the cerebellar cortex can be considered as divided into multiple layers. One model of cerebellar folding used a trilayer model of differential growth [117]. 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.

At folding initiation, the cerebellum closely mimics a bilayer system with the rapidly expanding EGL covering the core. The onset of folding is correlated with a differential expansion of these layers. However, the tissue mechanics of the developing cerebellum, such as tissue stiffness, the tensile profile, and the out-of-phase layer thickness, do not align with requirements for models based in elastic buckling [87] (Fig. 6a). As the progenitors in the EGL are dynamic and continually moving within the EGL prior to their radial migration, this layer may have fluid-like properties [87]. A model incorporating this fluid behavior of the EGL and the tensile profile of the developing cerebellum with the differential expansion between the EGL and the underlying tissue is able to capture the unique shapes seen at folding initiation [87, 118]. Tissue tension and its potential roles within folding and gyrification has been revisited and recently reviewed [119].

How individual granule cell behaviors coalesce to create emergent tissue-level structures remains unclear. Disruptions to proper chromatin remodeling in granule

cell progenitors alters cell division angle of granule cells and drives aberrant cerebellar folding [120]. Yet, disrupting kinase activity in granule cells leads to improper folding without any underlying change in the cell division angle [121]. Fascinatingly, the improper folding seen with the change in cell division angle is not aligned in the anterior-posterior axis but is aligned in the medial-lateral axis [120]. This would suggest a change in the direction of the underlying differential expansion and resulting forces. It is still an open question of how cell division angle informs tissue folding especially in light of the motility of granule cell progenitors within the EGL.

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 during evolution to 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 minimizes the consequences of local damage in adults. Insight into the importance of the folding-based subdivisions of functionality will likely come from the variety of birth defects and other conditions that present with folding disruptions (reviewed in [122]). 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 the degree to which stem or progenitor cells in the developing or adult cerebellum can be coaxed to 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

Filippo Casoni, Laura Croci, Ottavio Cremona, Richard Hawkes, and G. Giacomo Consalez

Abstract This chapter explores the mechanisms that regulate Purkinje cell (PC) 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

Purkinje Cells 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 [reviewed

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recently in 1–3]. Cerebellar architecture is built around multiple Purkinje cell (PC) subtypes [4–9] – most notably zebrin II/aldolase C [10, 11], 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 12], interneuron neurites [13], and soma [reviewed in 14] and glial gene expression profiles, for example, 5′-nucleotidase [15].

In the mouse, the general timeline of events that leads to cerebellar maturation from its embryonic *anlage* has been fully clarified [16–21]. Here we discuss some of the major features of cerebellar development, focusing on the ontogenesis of 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 22]. 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 [23–26], 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 migration impairs granule cell clonal expansion, and cerebellar foliation / PC migration failures result is a lissiform adult cerebellar cortex: for example, the naturally occurring mouse mutant *reeler* [(Relnrl): reviewed in 27].

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 [28, 29], and terminate with a spatial organization that parallels the pattern of PC stripes [30]. 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 19, 31]. 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 12], and interneuron neurites [13, reviewed in 14] and spontaneous and engineered mouse mutants with disrupted PC stripes have complementary alterations in the spatial arrangement of afferent terminals [32–34].

Cerebellar Anlagen and Germinal Zones

The cerebellum arises from a specialized region at the midbrain/hindbrain boundary [35–37]. In the mouse, at E8.5, the antagonistic interaction that takes place between homeobox genes *Otx2* and *Gbx2* defines the isthmic organizer region [38, 39], which controls the development of cerebellar structures via the secreted morphogens FGF8 and WNT1 [19, 40, 41]. 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 [18]. Importantly, however, homotopic and isochronic quail-chick grafting experiments have clearly shown that the caudal part of the early mesencephalic vesicle generates the rostral and medial part of the prospective cerebellum [35, 42–45]. 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 alar portion of the mesencephalic vesicle [42].

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: the rhombic lip (RL), located at the outer aspect of the cerebellar plate, adjacent to the roof plate (RP, dorsal), and 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 [46], and atonal homolog 1 (*Atoh1*) in the RL [47]. Cerebellar radial glial progenitors [48] fated to generate all GABAergic neurons of the cerebellum express *Ptf1a*, including PCs and all inhibitory interneurons – cerebellar nuclear interneurons plus basket, stellate, Golgi, and Lugaro cells in the cerebellar cortex [46, 49, 50]. Homozygous mutations of *PTF1A* are associated with cerebellar agenesis in humans [51]. Conversely, all glutamatergic lineages – the large projection neurons of the cerebellar nuclei, unipolar brush cells and granule cells – derive from *Atoh1*$^{+}$ progenitors [52–57]: their development is exhaustively reviewed elsewhere [22].

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 [58]. 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 [58]. 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 [59, 60]. Notch1 in the cerebellar primordium interferes with BMP2/4 signal transduction causing downregulation of the BMP target *Msx2*.

As shown by birthdating studies, cerebellar projection neurons, GABAergic (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 [18, 61, 62]. 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 [18, 20, 63].

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*$^+$ [46], *Ascl1*$^+$ [64] progenitors according to a two-step sequence [20, 22]. 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 to populate the nascent PC plate. The GABAergic interneurons from a different lineage [e.g., 65] 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) [66]. 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. Other recently identified factors have been implicated in this fate choice and subsequent ones [63]. PC precursors, after leaving the cell cycle, start migrating and populate different regions of the cerebellar cortex according to their birthdate [18, 55]. Instead, actively proliferating interneuron progenitors, 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 [67, reviewed in 68].

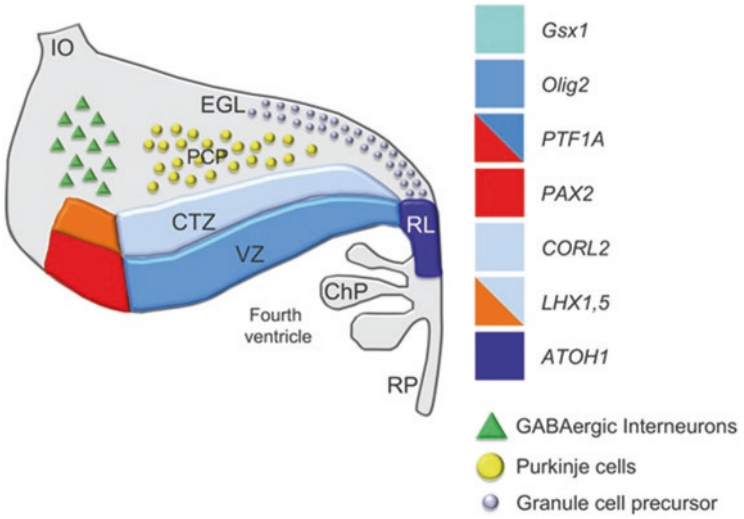

Fig. 1 A simplified representation of gene expression and cellular domains present in the E12.5 murine cerebellar primordium and giving rise to the mature cerebellar cortex. No reference is made here to cerebellar nuclei and their precursors. The drawing represents a sagittal section of the cerebellar 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 cortical 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$^+$ postmitotic 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

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 [69] or no effect on PC number. However, a null mutation of both *Olig2* and *Olig1* produces a reduction of committed PC precursors [49]. 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 [49]. However, this view is challenged by the results of short- and long-term lineage tracing studies performed by other authors [69], 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. Recently, the role of the OLIG transcription factor family in PC specification has been expanded by the recognition that OLIG3, expressed in the ventricular zone, restricts Pax2 expression, and thereby suppresses PC differentiation [70].

Importantly, the results of recent single-cell sequencing studies are casting new light on the transcriptional programs controlling cell fate specification of populations arising from the VZ and the RL, uncovering new markers, gene-expression cascades and neuronal subpopulations, and a number of previously unknown subpopulations that may play uncharacterized roles in cerebellar neurogenesis [71].

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 [72]. When OLIG2^{+} PC progenitors exit the cell cycle, they activate the expression of *Corl2* [73], which encodes a transcriptional repressor [74], and that of *Lhx1* and *Lhx5* [75], encoding LIM homeobox domains. However, unlike CORL2, LHX1, and LHX5 label delaminating interneuron precursors as well as postmitotic PC precursors [49, 73]. Cells co-expressing LHX1/LHX5 [75] and CORL2 [73] are differentiating VZ-born precursors committed to a PC fate.

Other PTF1A targets are expressed in the VZ in addition to those described above [76]. The *Drosophila atonal* homologs *neurogenin 1* (*Neurog1)* and *neurogenin 2* (*Neurog2)* are proneural genes encoding basic helix-loop-helix transcription factors. *Neurog1*$^{+}$ progenitors give rise to inhibitory cortical interneurons and some PCs [77, 78], 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 [79]. However, NEUROG1 and NEUROG2 are not required for the adoption of a PC fate [R. Hawkes, unpublished observation, and 79]. Interestingly, cell cycle analysis conducted by cumulative S-phase labeling on *Neurog2*^{CreERT2} knock-in 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 [79].

Purkinje cell neurogenesis is also controlled by *Zfp423*, encoding the homonymous Zn finger transcription factor, and orthologous to human *ZNF423*, implicated in rare cases of Joubert syndrome. *Zfp423* regulates the mode of cell division in a domain-specific fashion. A central domain of the protein is required for the

maintenance of the stem cell progenitor pool. Moreover, this factor controls DNA damage repair in the cerebellar VZ: a defective response to DNA damage causes a delay in cell cycle progression, contributing to the vermis hypoplasia and profound depletion of PCs observed in *Zfp423* mutants [80].

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 [10]; PLCβ3/4 [81]; HSP25 [82]: reviewed in [9]). The embryological origins of PC heterogeneity and pattern formation are only slowly coming into focus [83]. PC subtype phenotype is cerebellum-intrinsic and independent of neural activity (e.g., [84]) or afferent innervation [85, 86]. Cerebellar compartmentation appears to start at ~E10 in the VZ of the fourth ventricle but likely not sooner [e.g., 87, 88–90]. The first stage likely occurs when PCs undergo terminal mitosis between E10-E13 [61] in the *Ptf1a* expressing progenitor domain of the VZ [46, 76]. 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^{-} [91, 92]. However, individual PC stripes do not have a clonal origin [89]. 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., 91, 93–95].

Several regulatory genes are implicated in PC progenitor development. Among them, *Early B-cell factor 2* (*Ebf2*) [96] 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 97, 98]. *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 towards 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 [99]. 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 [100], dies by apoptosis [101, 102]. *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 [102]. Finally, some of the *Ebf2* null PCs that survive lose their PC subtype specification features and transdifferentiate into zebrin II^{+} PCs – the only genetic manipulation thus far shown to subvert PC subtype specification [101]. In fact, *Ebf2* acts to repress the zebrin II^{+} phenotype in late-born PCs [94]. 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 [103] 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, for example, the HSP25$^{+/-}$ distinction within the zebrin II^{+} family [82] have not yet been explored.

The First Formation of PC Afferent Projections

The main afferents to the cerebellum are climbing fibers from the inferior olives and mossy fibers from multiple sources, and their patterns of development are elaborate [reviewed in 104]. The earliest afferent projections to the cerebellum are mossy fibers (MF). In the adult, MF afferents terminate in the granular layer as glutamatergic synapses on the dendrites of the granule cells. The terminal fields of the MF are highly topographically organized into stripes aligned with overlying PC stripes [reviewed in 1, 8]. The role of PCs in organizing the MF axons is complex. During embryogenesis, many MF afferents enter the cerebellum at a stage at which the granular layer is not yet present. Intriguingly, they synapse ectopically on the PC somata. During the same interval, the MF also send collateral branches to the cerebellar nuclei. Presumably, the ingrowing growth cones recognize PC and CN subtypes, and thereby guide the formation of the adult striped topography, for example, ephrins and ephrin receptor tyrosine kinases, in particular ephrins A2 and A5 [105]. Subsequently, once postmitotic granule cells begin to migrate ventrally from the external granular layer through the PC layer to form the mature granular layer, beginning at an early postnatal stage [reviewed in 106], MF axons detach from the PC somata and synapse with the transiting granule cells. The earlier development is equally surprising. The very first MF afferents to enter the cerebellar anlage – beginning at E9 – derive from transient axons of the trigeminal ganglia [107]. Surprisingly, the first targets of the trigeminal ganglia axons are not the PCs but the cerebellar nuclei! Collateral projections are only observed to the PC plate a day later. Whether this developmental target sequence – first CN, then PC, then granule cell – is unique to the trigeminal ganglion afferents or applies to other MF is unknown. Likewise, because the trigeminal ganglion axon projection is only transient, perhaps its role is to serve as a pioneer, to pilot subsequent early afferent topography.

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 [9, reviewed in 92, e.g., Fig. 2 108, 109–111]. These

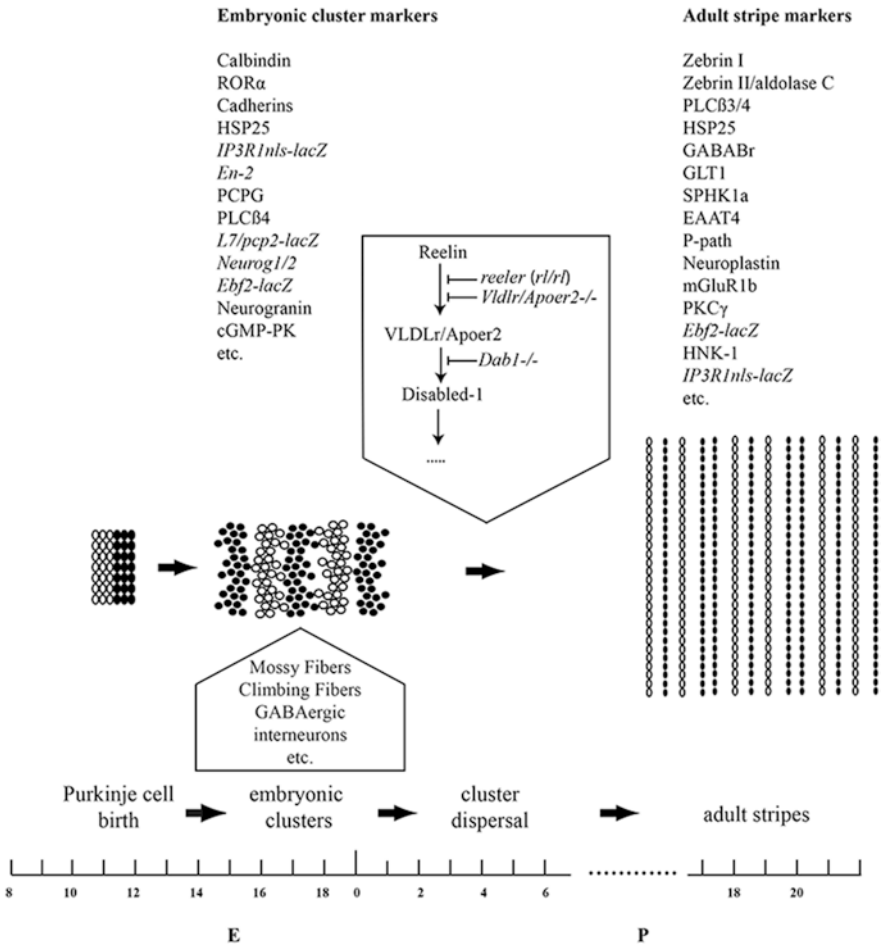

Fig. 2 From clusters to stripes. Embryonic clusters condense by migration from the cerebellar plate between E14-E18 (mouse). At this stage, numerous expression markers reveal that the PC population is already heterogeneous (exactly how many distinct phenotypes are present is not known, in part because 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 10 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 [9]

clusters are the targets by which cerebellar afferents and many interneurons become topographically ordered [reviewed in 8, 31]. The mechanism that converts the PC plate into the elaborate array of embryonic PC clusters – >50 are recorded [111, 112] – is not well understood. As PCs migrate towards 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 [113]. 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 VZ, (e.g., some version of the protomap model proposed by Rakic for the neocortex) [reviewed in 114], 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 [115], pointing to cell-cell adhesion molecules as possible organizers: cadherins [reviewed in 116] and integrins [e.g., 117] 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 [90]; 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. We focus here on the PC compartmentation but similar and aligned boundaries are also prominent in the granular layer [reviewed in 106]. 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., 110, 118–120]: the anterior zone [AZ: ~ lobules I–V {reviewed in 121}], central zone anterior (CZa: ~lobule VI) and posterior (CZp), the posterior zone [PZ: ~lobules VIII–dorsal IX: reviewed in 118], and the nodular zone (NZ: ~lobules ventral IX–X). Each transverse zone 1 into a reproducible array of parasagittal stripes (e.g., revealed by using zebrin II/aldolase C – [10, 11]: for zebrin II+/− stripes, these are labelled P^{+} and P^{-}: e.g., zebrin II – [119]; phospholipase (PL) Cß3/4 – [81]); the small heat shock protein HSP25 – [82]; or *L7/pcp2-lacZ* transgene expression – [reviewed in 9, 122].

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 [reviewed in 110, e.g., calbindin – 122] 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., 123], *Lurcher* Grid2Lc-J [124], and *cerebellar deficient folia* [125]; in others – for

example, the BETA2/*NeuroD1* null [126] – 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 [127]. The CZa/CZp boundary [128] is a perinatal restriction boundary for FoxP2 [110], *Gli* [109], and HNK-1 expression [129]. The CZp/PZ that separates lobule VII from lobule VIII is revealed in the perinatal cerebellum by FoxP2 [110, 112], PLCβ4 [130], and HSP25 [131] expression is associated with a phenotypic abnormality in the *lurcher* (*Grid*^{Lc}) mouse [124]. 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 [109] and FoxP2 [110]. A granular layer transverse boundary in embryonic stem cell chimeras is also located at around the PZ/NZ boundary [127].

Starting at around E18, the embryonic clusters transform into adult stripes triggered by RELN signaling [reviewed in 132, 133]. 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 [132] and binds to two PC receptors – the apolipoprotein E receptor 2 (Apoer2) and the very low-density lipoprotein receptor [VLDLR: 134, 135]. Both receptors are required for normal stripe formation, and if RELN is absent (e.g., the *reeler* mouse), PC cluster dispersal is blocked, and the adult mouse retains the embryonic cluster morphology and is ataxic [reviewed in 27]. RELN binding induces Apoer2/Vldlr receptor clustering [136], which triggers a protein kinase cascade and tyrosine phosphorylation of the docking protein Disabled1 (DAB) [137–141] by Fyn and Src [139, 142], 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 [143]. 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 [144]; divergent roles are also seen in the developing cerebral cortex [145]. Similar behavior is seen in several naturally occurring mutants. For example, *meander tail* [mea2J: 146], *rostral cerebellar malformation* [Unc5crcm: 123], and *cerebellar deficient folia* [Ctnna2cdf: 125] 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 [131]. The GIRK2 protein mutated in *weaver* [147] 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, for example PLCß4 [130]; an IP3R1 promoter-nls-lacZ transgene [148]; FOXP2 [112], but others are only expressed in stripes at one stage or show very different expression patterns perinatally versus the adult, for example, HSP25 [e.g., HSP25 131], lysosomal acid phosphatase 2 [149]. There is limited evidence of the continuity of the cerebellar topographical 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., 92, 112, 148]. 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 PLCß4 expression [130]. 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

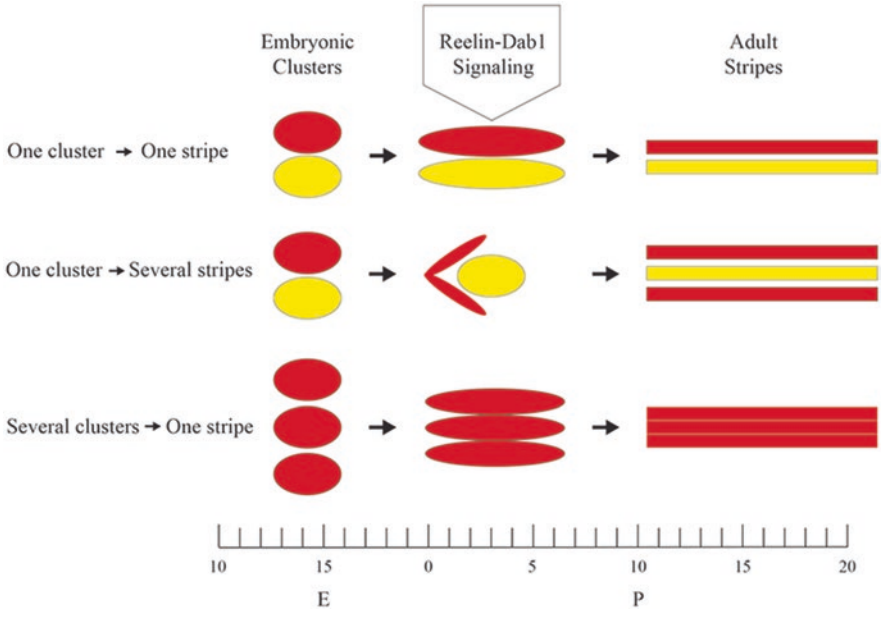

Fig. 3 Three models for the transformation of embryonic PC clusters into adult stripes: one embryonic cluster forms one adult stripe; one cluster splits to yield several stripes; or several clusters combine into a single stripe. All three models are found

terminate in different sub-stripes [150, 151] and in the expression of an *L7/pcp2-lacZ* transgene [122]. A similar covert heterogeneity is seen in ostensibly homogeneous zebrin II$^+$ stripes when co-labelled for HSP25 [82]. 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 [31].

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 and postnatal period [152, 153]. 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 [154]. 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 [155]) and *Bax−/−* mice have a 30% excess of PCs over controls [e.g., 156]. 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-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 [157]. Equally striking, recent evidence has linked PC development to the pathogenesis of autistic spectrum disorders [reviewed in 158]. In particular, selective deletion of the *Tsc1* gene in the PC lineage from conditional knock-out mice has been found to cause a decrease in PC number, increased spine density, and autistic-like alterations of social behavior [159]. One of many insults

thought to trigger autism is maternal fever [160]. Possibly related to the putative role of the cerebellum in autism, we recently found that immune activation and fever in pregnant mice between E13-E15 resulted in adult progeny with significantly wider zebrin II+/− stripes, greater numbers of PCs, poorer motor performance, and impaired social interactions in adolescence [161].

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 learnt 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 [162]. 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

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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

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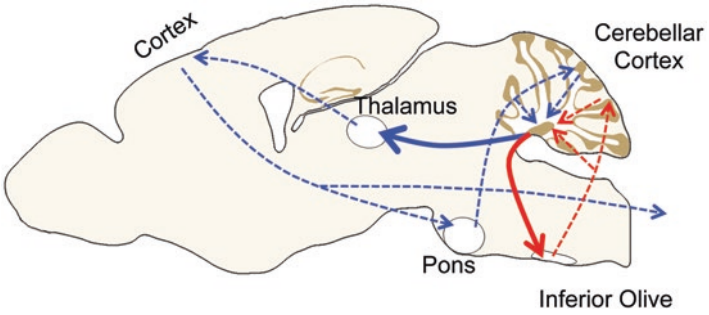

Fig. 1 The cerebellar nuclei are central to cerebellar circuitry. They lie at the centre of two cerebellar loops: the cerebello-thalamo-cerebro-cortical circuit (blue) which links the cerebellum back to the cerebral cortex and the olivo-cortico-nucleo-olivary loop (red)

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).

Recent single-cell RNA sequencing studies have revealed a simple modular cell type structure underlying the organisation of neurons into nuclei and their long-range connectivity. In combination with a temporal pattern underlying their developmental origins, a clear logic underlying their patterning and evolution is beginning to emerge. However, 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 five neuronal types.

Immunohistological and molecular techniques showed large projection neurons to be glutamatergic while GABAergic [6–8] projection neurons with very small soma project to the inferior olive (Fig. 2). Glycinergic neurons were found to project to the brainstem [9] or to the granule cell layer of the cerebellar cortex [3, 10–12]. Unlike the other CN cell types, these latter nucleocortical neurons are not

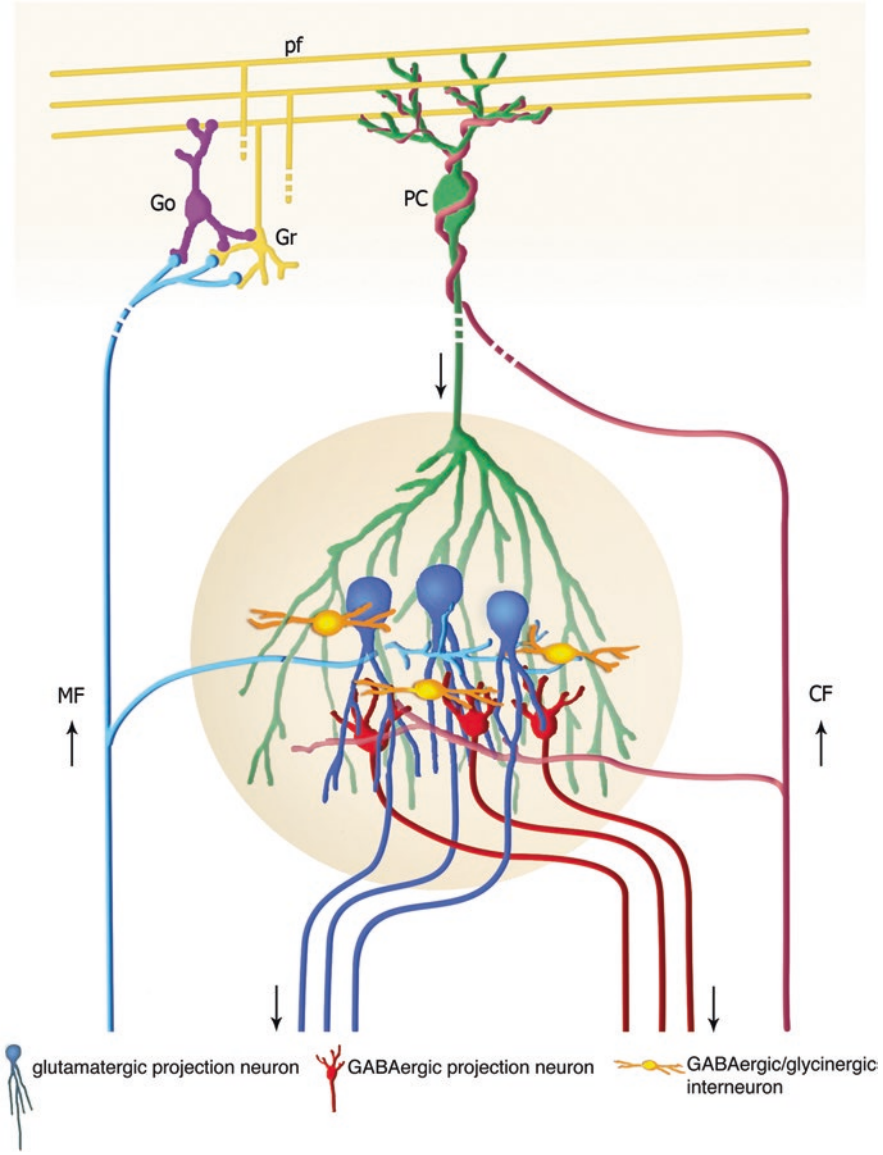

Fig. 2 The cellular composition of the cerebellar nuclei. Nuclei receive inputs from the Purkinje cells in the cerebellar cortex (green), as well as collaterals from the mossy fibres (light blue) and climbing fibres (pink) as they travel to the cortex. Within the nuclei, there are two types of projection 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

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].

Local interneurons have long been proposed to be inhibitory. Chan-Palay [4] noted small GABAergic neurons with fusiform or multipolar somas, limited dendritic trees and short axons. Similarly, candidate population of small soma glycinergic neurons which colocalise with GABA [14] in the interposed and lateral nuclei appear to target adjacent, presumptive glutamatergic projection neurons [15, 16],

Despite the fact that cells differ along both rostral-caudal and lateral-medial axes, such as a higher density of nucleo-olivary neurons in the ventral, lateral and interposed CN [17], models of cerebellar function have assumed a homogeneous allocation of each CN cell type to parallel the long-assumed homogeneity of cerebellar cortex [18, 19].

Recent single cell RNA sequencing experiments have largely confirmed and consolidated this spectrum of studies. Most importantly, they have identified that five basic cell classes are repeated across every CN and across chick, mouse and human [20]: Glutamatergic projection neurons (Types A and B), Sox14 expressing GABAergic nucleo-olivary neurons [21], local GABA+glycinergic interneurons (Type 1) and nucleocortical GABA+glycinergic interneurons (Type 2).

An important and exceptional class is a large soma, glycinergic, ipsilaterally projecting population in the medial nuclei [16] that appear to share much of the transcriptomic identity to a Class-B glutamatergic projecting neuron [20]. Intriguingly, in the medial nucleus, these inhibitory ipsilaterally projecting neurons sit side by side with glutamatergic, contralaterally projecting neurons that target the same regions of ventral brainstem and the ipsilateral ventromedial medullary reticular formation. This paired medial nucleus output, which might share a common developmental origin despite different neurotransmitter type, might regulate posture and balance through a system of cross-midline control, similar system to that of the vestibular control of horizontal eye movements [15].

A second significant variation in the homogeneity of cell class distribution is found in the human dentate nucleus, a hugely expanded lateral ribbon of cells that was presumed to be an expanded lateral nucleus. The dentate contains purely Class-B glutamatergic neurons and, uniquely, lacks a Class-A glutamatergic projection [20]. Since the dentate nucleus has a large input via the thalamus to the prefrontal cortex, this result suggests a selection for the Class A projection neuronal type contributes to the specialisation of this pathway in humans and great apes (which also display a dentate nucleus).

Five distinct types of CN neuron underlie the diversity of functional output of the cerebellum. Projection neuron activity is modulated by Purkinje cell inhibition while local interneurons play an as yet unknown role in shaping output. Important variations in exceptional cell types may underlie the regulation of posture and the cognitive cerebellum in humans.

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-cerebrocortical circuits which link the cerebellum back to different parts of the cerebral cortex [22]. Extensive labelling studies in the mouse using anterograde viral tracer show that while the medial nucleus is more distinct in its connections there is an extensive overlap in the projection patterns of the excitatory output of all three major cerebellar nuclei [20].

Inhibitory neurons derived from a Sox14 pool of precursors [21] send predominantly contralateral projection to the inferior olive [21, 23, 24] and are key components of the olivo-cortico-nucleo-olivary (OCNO) circuit. This is 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 [25].

Efferents show widespread and overlapping projections with the exception of the nucleo-olivary GABAergic axons that descend to hindbrain. The origins of the diversity and the mechanisms underlying the targeting of axons are largely unexplored. These questions are 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 modulate cerebellar output by influencing the spontaneous baseline firing rate of CN neurons [26, 27]. 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 from the hemispheric PCs [28]. 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 [29].

While both PCs and CN neurons are spontaneously active [30, 31], 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 [32–34]. When PC and CN neurons are monitored simultaneously, they do not give the expected reciprocal firing rates that would result from PC inhibition [35–38]. Instead, CN neurons are extremely sensitive to the synchronous activity of PC

inputs [39] suggesting that the development of a mapping of PC populations into the CN is a critical factor in cerebellar function.

In addition to afferents from the PCs, the CN also receive branches from mossy fibres (MFs) and climbing fibres (CFs). These send signals directly to the CN, bypassing cerebellar cortical processing [28]. 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 [24, 40, 41]. In contrast, the MF collaterals to the CN are bilateral and show a looser zonal organisation [28, 42]. 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 [29, 43, 44].

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 [45, 46] with inputs targeting both glutamatergic [47, 48] and GABAergic projection neurons [8]. However, since the PC axonal target field is wide and conical [49], it is estimated that each PC can encompass tens of CN neurons complicating a simple explanation of convergence. Similarly, the proximity of axons' terminations to the soma of CN neurons is likely to be of considerable significance in determining synaptic strength [16]. 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 [50], which may comprise the glycinergic, nucleocortical 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 midbrain and hindbrain and identified the cerebellar anlage within the dorsal part of rhombomere (r)1 of the hindbrain [51–53]. 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 [54]. Ptf1a is expressed in the dorsal ventricular zone of r1 and characterises progenitors of GABAergic cells [55]. The boundary between the ventricular zone and the dorsal roof plate is known as the rhombic lip [56] and expresses Atoh1 [57]. This highly proliferative zone of Atoh1 expression gives rise to glutamatergic cerebellar neurons [58, 59].

Birth-dating has shown that some neurons within the CN are among the first-born cell types of the cerebellum [60]. Experiments using either BrdU or a replication defective adenovirus [61] 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–E12.5 [21, 62] and appears to be regulated by a common temporal signal [63]. However, the allocation of GABAergic versus glutamatergic fate is strictly a property of progenitor position within either a Ptf1a- and Atoh1-positive pool [55, 58, 59, 63, 64].

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 [65]. A detailed analysis of postmitotic precursors of CN neurons identified the expression of the transcription factors Lhx2/9, Meis1, Meis2, and Irx3, as well as genes that are not frequently used as markers in development: Gja9, Mbd2, Htr3a, and Girk4 [66]. Subsequent analysis showed that Meis2 co-expresses with Lhx2/9 in glutamatergic

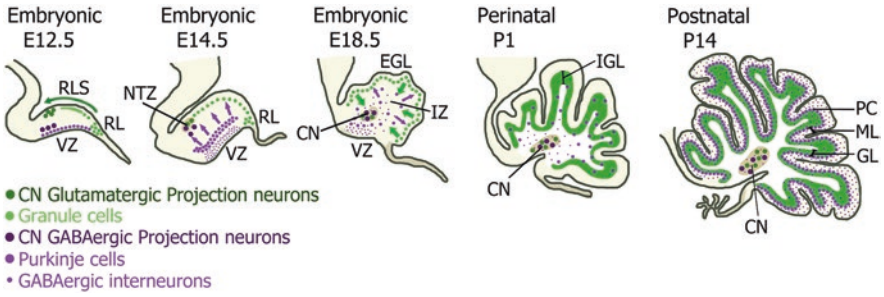

Fig. 3 The developmental timeline of the cerebellum, depicted in sagittal view. GABAergic neurons are derived from the ventricular zone (VZ) while glutamatergic neurons arise at the rhombic lip (RL). The cerebellar nucleus projection neurons are the first born from both progenitor zones, preceding first 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

projection neurons of the lateral CN derived from the rhombic lip [59], while Irx3 may instead represent a separate population of neurons, likely the GABAergic nucleo-olivary neurons [67].

Glutamatergic projection neurons represent the first cohort in a sequence of neurogenesis from the rhombic lip that ends with the generation of granule cells [51, 58, 59]. A separate domain of Atoh1 expression at the midbrain-hindbrain boundary gives rise to earlier born extracerebellar neurons [68]. At the rhombic lip, lateral and then medial CN are produced in discrete temporal waves [69, 70]. CN neurons actively migrate from the rhombic lip in a subpial layer guided by diffusible netrin and slit proteins [71, 72] and sequentially express Pax6, Tbr2, Tbr1 and Lmx1a [67, 73]. As the postmitotic neurons enter the NTZ, Tbr1 and Tbr2 are upregulated and Pax6 is downregulated [73]. In the absence of Pax6, rhombic lip-derived CN neurons are absent from the cerebellum [67]. 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

Our recent studies of mouse development showed that GABAergic nucleo-olivary neurons of the lateral and interposed nuclei are derived from the Sox14 lineage in the cerebellum [21] . It is assumed that they are born in 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 arise in a discrete, early temporal window of cell production (E10.5–E11.5 in mouse) [21] followed by other GABAergic interneurons [74]. In contrast to these later born cell types, both PCs and GABAergic CN neurons express Neurog2. Irx3 immunopositive cells are evident in the ventricular zone 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 [66, 67]. 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

Ventricular zone progenitors require the expression of Ptf1a for GABAergic specification, rather than defaulting to a granule cell fate [55, 64]. Within the Ptf1a ventricular zone, combinatorial gene expression demarcates discrete germ zones that are thought to give rise to the different types of interneurons [66, 74–80]. 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 ventricular zone [81] 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 [81, 82], including Neurog1 (Ngn1)-positive interneurons of the CN, which are born at E17.5 in mouse [83]. Mutation of PC progenitor transcription factors Olig2 and Gsx1 disrupt the production of Pax2 lineages suggesting that the latter is derived from the former in development [84]. 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 requires that cells recognise each other and assemble nuclei distant to their origins. A clear waiting period at the NTZ when GABAergic projection neurons remain segregated from glutamatergic neurons indicates that this assembly is an active and temporally regulated process [21]. However, 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 [71, 72] (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 [69, 71]. 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 [69] or Tbr1 knocked down in mouse [73], 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 [62, 65].

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 [73].

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 [85]. There is some debate over whether an organism is considered to have cerebelloid structure if they lack CN, since it is these cells that form the dominant output [86]. 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 [87, 88].

The replacement of CN by eurydendroid cells appears to be a ray-finned fish adaptation as there is evidence for a single CN in the shark [89]. CN are absent in Lampreys, where the cerebellum is both structurally [90] and transcriptomically [91] 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 [92].

Like sharks, anamniote amphibians have a single CN [90]; however, the number and diversity of CN dramatically increases in amniotes. The diversity of subnuclear compartments makes definitive designation of cerebellar nuclei somewhat unreliable. It is broadly accepted that there are two major CN in birds [20, 93] and three major sets of CN in rodents: the medial, interposed, and lateral [20, 94, 95]. 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 [16]. This systematic variation in organisation suggests that comparative studies may offer an important insight into how a common repertoire of CN neuronal types is adapted to build a diversity of CN structures.

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 [59, 96]. GABAergic neurons share a progenitor transcriptional profile with auditory nuclei and, perhaps most prominently, the inferior olive [55].

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 [97], 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 [98] 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 in regard to understanding both the types and the development of the CN neurons. Despite this, some key questions about the specification and lineage of CN neuronal types remain unanswered. A defining feature of development is that cells transit through the NTZ, yet nothing is known of the factors that regulate nucleogenesis. 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 [45, 118].

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 [69, 73].

Finally, how the variable distribution of the five basic CN cell types across a diversity of cerebellar nuclei is specified, and how they develop a network of intranuclear connectivity are key developmental questions. Given that different densities of the same 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 it is patterns of connectivity and plasticity that are key to generating an assortment of functions. The answers to these questions will be of huge significance for functional models of the cerebellar network. They 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 both how the cerebellum works and its clinical vulnerabilities.

Table 1 Cerebellar disorders exhibiting nuclear pathology

Disorder	Aetiology	Pathology	Clinical features	References
Zellweger (cerebro-hepato-renal) syndrome	Autosomal recessive disease caused by mutations in PEX genes. Migration failure from14 weeks of gestation in humans	Dysplasia of the dentate and olivary nuclei (DOD), as well as cerebellar hypoplasia and migrational defects PCs	Developmental delay, seizures and EEG abnormalities, as well as generalised hypotonia, renal cysts, and joint calcifications	[99–101]
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.	[102, 103]
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	[104–106]
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, mild to severe mental and motor developmental delays	[62, 107–109]
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, there 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.	[110–112]

Disorder	Aetiology	Pathology	Clinical features	References
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.	[113, 114]
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	[115–117]

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The Role of Non-coding RNAs in Cerebellar Development

Maryam Rahimi-Balaei, Miguel Ramirez, Ishita Gupta, and Daniel Goldowitz

Abstract Since the sequencing of the human and various mammalian genomes, it is clear that there are far too few protein-coding genes to specify and coordinate the complex series of developmental events that results in a mature brain. Non-coding RNAs (ncRNAs) are seen as a fount of transcriptional richness that can regulate gene expression in time and space. Together, protein-coding RNAs and ncRNAs can reproducibly replicate a formed and functioning brain. In this chapter, we focus on the roles of three dominant species of ncRNAs – enhancers, long non-coding RNAs, and microRNAs – in driving the development and function in the mouse cerebellum.

Keywords Non-coding RNAs · Enhancer RNA · MicroRNA · Long non-coding RNA · Cerebellum · Gene regulation · Development · Mouse

Introduction

Our laboratory's enduring interest is in the genetic architecture of brain development with the cerebellum as the proxy for the overall brain. We have approached this from the analysis of single gene mutations in mice [1] to whole transcriptomes using microarray technology (http://www.cbgrits.org/) [2]. However, we are acutely

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aware that much of gene regulation, particularly in development, occurs via the ~98% of the genome much of which is populated by DNA that specifies non-coding (nc) RNAs [3]. To approach this relatively unexplored area in brain development, we were invited to join the RIKEN FANTOM5 project (http://fantom.gsc.riken.jp/5/) where we submitted replicate mouse cerebellar samples (as we had done for cbGRiTS [2]) over embryonic ages, every 24 h from embryonic day 11 (E11) to E18 and every 72 hours from postnatal 0 to postnatal day 9 [4].

The FANTOM5 consortium used HeliScopeCAGE technology which combines the Cap Analysis of Gene Expression (CAGE) protocol and next-generation Helicos sequencing to produce direct, high-precision measurement of transcription based on 5′ end sequence of mRNA [5]. Using their deepCAGE technology, we were able to see a full genome read-out of transcription start sites, not only for protein-coding regions, but the far more abundant non-coding regions of the genome as well [6].

The FANTOM5 dataset is a rich source for defining ncRNAs. Several high-profile publications have provided atlases of enhancer(e)RNAs [7, 8], micro(mi) RNAs [9] and long non-coding(lnc)RNAs [10]. The emerging picture of these ncRNAs is that they are developmentally relevant, particular to the brain. Now with deepCAGE data, we have an unparalleled look at the role of non-coding elements and mammalian cerebellar development.

Given the data that has come from FANTOM5, we have a unique opportunity to explore the role of ncRNAs in cerebellar development. The cerebellum (or "little brain") is an excellent placeholder for the brain at large – with a limited number of cell types, defined epochs of development for each of the cell types, and unusually large numbers of granule cells (it has been estimated that granule cells compose about 50% of the neurons of the developed mammalian brain; a great resource for biochemical and molecular studies). Previously, we had taken a gene-at-a-time approach and made nice headway with specific genes such as Atoh1 [11] and Pax6 [12]; but with the advent of major advances in high-throughput microarray and fluidic technologies, bioinformatics and functional genomics, a collaborative group of a few labs can do large-scale analyses to reveal the function of ncRNAs. From a human health point of view, it is clear that statistically significant signals from Genome Wide Association Studies (GWAS) identify disease loci that largely point to non-coding parts of the genome [13] and of particular relevance are developmental neurological conditions (e.g., autism and schizophrenia) [14]. FANTOM5 work has found that disease-associated SNPs are more common in regulatory regions than in exons [7] and an "enrichment of conserved lncRNAs" are highlighted in GWAS traits [10]. Other recent work has implicated lncRNA [15] and miRNA [16] in autism spectrum disorder.

In this chapter, we explore a substantial part of the 98% of DNA, much of which provides the template for ncRNAs. We focus on the three major types of ncRNAs where there is mounting evidence for their importance in brain development and disease. We hope that this chapter will encourage further studies on ncRNAs (and their cognate DNA sequences) which will be important to understanding gene regulation in cerebellar development and providing candidate targets to treat

neurodevelopmental abnormalities. This will set the stage for mechanistic approaches to study how ncRNAs control the development of neural tissues.

Enhancer RNAs

The transcription of ncRNAs, termed enhancer RNAs (eRNAs), has typically been found to occur at enhancer elements. Enhancers are non-coding regulatory sequences located distal to promoters that serve as binding sites for transcription factors (TFs) and result in the activation of target gene expression. Enhancers function as regulators of tissue-specific and cell type-specific gene expression during brain development [17]. These sequences serve as docking sites for neural-specific lineage-defining TFs which regulate cell identity and differentiation [18–20]. eRNA transcription is highly correlated with markers of enhancer activity such as enhancer-associated post-translational histone modifications (H3K27ac and H3K4me1), open chromatin conformation, TF binding and the recruitment of transcriptional cofactors [7, 8, 21–23]. The expression eRNA is also positively correlated with the level of transcription at proximal promoters and putative target genes. In the context of brain development, eRNA transcription is highly tissue-specific and can serve as a marker of cell state [8]. Transcribed enhancer (TE) elements are enriched for cell type- and temporal-specific transcription factor binding sites of key regulators of cell differentiation and specification [7]. The importance of these eRNAs in human health and development is highlighted by the enrichment of disease-specific variants within these sequences from a broad range of diseases including psychiatric and neurological disorders [24]. Collectively, this evidence supports the view that eRNA transcription is a robust signal of enhancer activation and plays a role in the regulation of gene expression in the brain.

During cerebellar development, enhancer sequences exhibit temporally specific activity and regulate the expression of genes critical for various stages of neuronal development [25–27]. Previous examinations of enhancer activity genome-wide have identified temporally specific open chromatin regions between postnatal and adult cerebella and verified that many of these regions function as neuronal enhancers using H3K27ac chromatin immunoprecipitation followed by sequencing (ChIP-seq), reporter gene assays and CRISPR-mediated activation [25]. Additionally, the open chromatin landscape has been assessed during embryonic and postnatal cerebellum development using single-nuclear ATAC-seq (snATAC-seq) which provided a comprehensive atlas of predicted regulatory sequences at cell-type resolution [26]. Recently, our lab has provided a novel view of active enhancers during embryonic and early postnatal development using ChIP-seq of enhancer-associated histone marks H3K27ac and H3K4me1 [27]. Our study identified developmental enhancers with activity temporally specific to embryonic or postnatal development. These enhancers were predicted to regulate target genes by correlating H3K27ac ChIP-seq signal with gene expression. Clustering of gene targets revealed spatially restricted expression patterns, indicating cell type-specific expression regulation. Functional

analysis of these target genes indicated that enhancers regulate processes spanning several developmental epochs such as specification, differentiation and maturation. The results of these analyses were utilized to discover TFs with novel functions in the context of cerebellar development. For example, Bhlhe22 is a target gene predicted to be downstream enhancers with temporally specific activity during postnatal development. Using immunofluorescent staining, we identified Bhlhe22 expression in postnatal differentiating granule cells migrating from the external granular layer (EGL) to the internal granular layer (IGL). Knockdown of Bhlhe22 expression in primary granule cell cultures revealed that Bhlhe22 regulates postnatal granule cell migration. As this dataset is a valuable resource to the cerebellar research community, the results of our analysis is easily accessible online in the Developmental Cerebellar Enhancer Atlas (https://goldowitzlab.shinyapps.io/developing_mouse_cerebellum_enhancer_atlas/), where our dataset can be queried, curated and exported by the cerebellar research community.

In the context of the transcribed enhancers (eRNAs) in the developing cerebellum, they have been quantified using cap analysis of gene expression followed by sequencing (CAGE-seq) as part of the FANTOM5 project. CAGE-seq, which detects newly transcribed or nascent RNA, is typically used for quantifying eRNAs genome-wide, as they are expressed at low abundance (relative to genes) and are relatively unstable and have a high turnover rate. The FANTOM5′s large-scale effort found that neural tissues and neurons have a high abundance of cell-specific enhancer transcription [7]. Indeed, studies in neurons are prominent among those contributing to our understanding of enhancers and eRNA [22, 28–31]. Our lab took part in this effort, submitting mouse cerebellar samples from 12 stages throughout embryonic and early postnatal cerebellar development.

We have conducted an analysis of this cerebellar CAGE-seq eRNA dataset, with the goal of identifying transcribed enhancers active during cerebellar development and characterizing the molecular mechanisms they regulate during embryonic and early postnatal stages. In combination with enhancer-associated histone modifications H3K4me1 and H3K27ac, we identified 1665 robust cerebellar transcribed enhancers and their respective eRNAs. Many of these cerebellar transcribed enhancers overlapped with cerebellar enhancers identified in previous studies as well as with enhancer sequences validated using transgenic reporter assays from the VISTA enhancer database. Temporal analysis of eRNA transcription, as a proxy for enhancer activity, revealed clusters of enhancers that peak in activity during either embryonic or postnatal stages, highlighting an importance for temporally specific events. A comparison with tissues from the FANTOM5 database suggested that robust cerebellar TE transcription is specific to the cerebellum. This indicates that eRNA transcription may be critical for fine-tuning expression for developing cells in the cerebellum. Putative gene targets were determined by correlating TE transcription with expression of cis-located genes. Functional analysis of putative target genes identified that TEs were enriched for neurogenesis, neuronal differentiation, neurite growth and synapse development. In comparison to non-transcribed enhancers (H3K4me1+ and H3K27ac+), TEs were more highly enriched for biological processes specific to cells in the developing brain; while non-transcribed enhancers

regulate genes more highly enriched for nonspecific constitutive processes. The expression of eRNAs were validated for a transcribed enhancer predicted to regulate Nfib, a gene critical for the postnatal differentiation of granule cells. Fluorescent in situ hybridization of eRNAs transcribed from putative Nfib enhancers identified colocalization with Nfib transcripts and expression within differentiating granule cells. Overall, our investigations identified cerebellar TEs with temporally and tissue-specific transcription and the results of our analysis suggest that TEs regulate processes critical for neurogenesis and differentiation the development of the cerebellum.

Given that previous studies have identified a tight association of eRNA transcription with putative target genes, future studies should focus on validating the expression of these eRNAs and their regulatory potential. eRNAs can be detected using FISH as a way of validating, sequencing data and identifying the cells in which they are transcribed, as demonstrated in the postnatal cerebellum [27]. To explore whether eRNAs are critical for the regulation of putative target gene expression, several studies have found success utilizing RNA interference, antisense oligonucleotides and genome editing to perturb eRNA function and measure ensuing phenotypes [32–35]. One important consideration is that eRNAs have been found to regulate genes in *trans,* emphasizing the need for RNA sequencing to identify downstream targets. Cerebellar-specific eRNAs, as identified in our recent analysis and by Yao et al., would be prime candidates for validation as their specificity suggests a regulatory role important for cerebellum development [31]. As transcribed enhancers and changes in eRNA expression have been associated with neurological and psychiatric disorders [24], characterising cerebellar eRNAs may provide insight on the genetic origins of cerebellar phenotypes in these disorders. Finally, given recent evidence that enhancer activity and open chromatin conformation is cell type-specific during cerebellar development [26, 27], single-cell sequencing of eRNAs could reveal a similar pattern for transcribed enhancers. The recent development of C1 CAGE has made this possible, which detects eRNA transcription start sites in single cells [36].

Many studies have identified the importance of transcribed enhancers in the context of cell specification and differentiation (myogenesis [37, 38], osteoclast development [39], T-cell and B-cell differentiation [40, 41], cardiac development [42] and embryonic stem cell differentiation [8]), thus identifying cell-specific eRNAs may provide further insight to the regulatory mechanisms driving the development of the various cell lineages in the cerebellum.

Long Non-coding RNAs

In recent years, sensitive RNA-based sequencing technologies have emerged to give rise to unbiased genome-wide transcriptomics, like ENCODE [43] and FANTOM5 [44, 45]. Such datasets offer an opportunity to study the ncRNAs involved in the

regulation of gene expression, like in the development of the central nervous system (CNS).

Among the many categories of regulatory ncRNAs, the class of lncRNAs remains largely heterogeneous and uncharacterised in their function. They are defined as long (>200 nucleotides) RNAs with no protein-coding potential. They are 5′ capped, poly-adenylated, undergo splicing and are derived from genomic regions that can be antisense, intronic, intergenic, or overlapping protein-coding loci. The proportion of non-protein-coding DNA seems to increase with developmental complexity [46]. LncRNAs have diverse interactions with DNA, RNA, and proteins which aligns with potential function in organizing and regulating cellular processes [47]. This has led to the idea that gene regulation by lncRNAs might have been important in giving rise to the diversity of cell differentiation programmes underlying development in multicellular organisms [48, 49]. As expression of mammalian lncRNAs shows greater tissue specificity than that of coding genes [50], it seems likely that they might contribute to tissue-specific regulation. As part of FANTOM5, studies by the RIKEN group have created a massive atlas of lncRNAs in humans [10] and again showed strong support for tissue-specific roles [6].

Expression of lncRNAs in the mammalian brain is impressive – it has been estimated that most of the lncRNAs are expressed in the mouse brain [47] and about 40% in the human brain [51, 52]. LncRNAs have been shown to be vital for neuronal differentiation, neuronal cell maintenance and neurogenesis [53]. Investigating the function of lncRNAs in brain development is thus an exciting direction.

Molecular patterning and regulatory pathways in CNS development have an important temporal component that dictates the sequence of events required for correct development. This stresses the importance of understanding the genetic underpinnings of critical time windows. To this end, transcriptomic expression data across a time course can help us capture gene regulatory elements like lncRNAs with developmentally crucial dynamics. The Goldowitz group participated in the international FANTOM5 consortium led by RIKEN to create a transcriptomic expression dataset for the cerebellum; whole cerebellar tissue was collected from 12 developmental timepoints (three biological replicates per timepoint) – embryonic days (E) 11.5 to E18.5 and postnatal (P) days 0, 3, 6 and 9 – and processed for cap analysis of gene expression (CAGE) sequencing [44, 54]. With CAGE, every RNA molecule that is 5′ capped is captured and sequenced at a single-nucleotide resolution towards its 5′ end. Once mapped back to the genome, this gives us not only the transcriptional expression levels, but also transcriptional start sites, or promoters, due to the 5′ sequence information [54].

To test the hypothesis that there is cerebellum-specific lncRNA involved in its development, we utilised the FANTOM5 time-course transcriptome to construct a catalogue of lncRNAs that are highly and specifically expressed in the cerebellum.

Out of a total of over 150,000 unique transcripts identified by the RIKEN FANTOM5 consortium across all tissue and cell samples, the subset that is robustly expressed in the cerebellar time-course samples consists of 16,138 unique transcript entries. To distinguish a subset of these transcripts that are identified as lncRNAs, we used the GENCODE atlas of annotated mouse lncRNAs (version M16)

consisting of 13,154 unique transcripts (removing splice variants). GENCODE assigns the biotype "lncRNA" based on a combination of factors – genomic location such as intergenic, intronic, or antisense together with the absence of an open reading frame (ORF), experimental data and/or literature showing no protein-coding power [55–57]. Overlapping this list of GENCODE annotations with the list of 16,138 expressed transcripts in the cerebellum time course, we identified 180 transcripts to be lncRNAs expressed in the developing cerebellum. Z-score is a widely used metric for tissue specificity [31, 58]. To capture cerebellum tissue-specific transcripts, Z-scores were generated per transcript based on the average expression of the transcript across all 399 mouse samples submitted to the FANTOM5 consortium, spanning 271 tissue types and 128 primary cell types, including the 12 cerebellar timepoints as independent samples [44, 59]. A transcript with a Z-score of 3 in E11.5 cerebellum, for example, would mean it is expressed in E11.5 cerebellum at a level that is three standard deviations above its most expected expression value across tissue types. Of the 180 lncRNA transcripts, only the ones having a Z-score $\geq$3 in at least one of the 12 cerebellar timepoints were retained; their high expression values at those timepoints being cerebellum-enriched with a p-value <0.003. A caveat of FANTOM5 is that the tissue types apart from the cerebellum are mostly non-neuronal, so we are unable to compare the cerebellum to other parts of the brain.

This analysis resulted in a list of 66 hits that can be ranked based on the average or age-specific expression levels, or alternatively the average or age-specific Z-scores (index of tissue-specificity). For our interests, this list was ranked in decreasing order of average expression level across the cerebellar time course (Table 1).

Based on a defined criteria, like expression levels and/or tissue-specificity in distinct time windows of expression, candidate lncRNA(s) can then be validated with spatial characterisation to give a cellular and molecular context to a putative function in cerebellar development.

The Universe of MicroRNAs

miRNAs in Cerebellar Development

The miRNAs are single-stranded ncRNAs with the length of 18 to 25 nucleotides [60, 61]. miRNAs, like other members of various classes of ncRNAs, are responsible for controlling a wide range of cellular functions such as apoptosis, cell proliferation, differentiation, metabolism, stem cell renewal and stress response [62, 63]. During miRNA biogenesis, primary (pri-) miRNAs are transcribed by RNA polymerase II from intergenic, intronic and exonic regions within the genome to produce a hairpin structure termed as pri-miRNA. Subsequently, during maturation of miRNAs, the RNase III enzyme Drosha and its double-stranded RNA-binding partner

Table 1 Highly expressed lncRNAs enriched during cerebellar development

S.No.	Name	Average Expression (TPM)	Z-score											
			E11.5	E12.5	E13.5	E14.5	E15.5	E16.5	E17.5	E18.5	P0	P3	P6	P9	Average Z-Score
1	p1.ENSMUST00000156068	196.47	2.59	3.16	3.02	2.63	2.24	2.21	2.02	1.75	1.10	0.68	0.52	0.01	1.83
2	p1.Miat	85.15	3.04	5.01	3.22	1.92	1.95	1.76	1.55	1.75	2.25	1.90	1.39	1.06	2.23
3	p1.Peg13	80.64	0.90	1.54	2.24	2.29	1.69	1.50	2.07	3.18	1.72	1.43	1.58	2.95	1.92
4	p1.C130071C03Rik	54.62	5.91	5.21	5.46	4.46	4.13	3.77	3.22	3.32	4.97	3.82	3.53	1.99	4.15
5	p1.Meg3	48.82	0.00	-0.14	0.40	0.59	1.87	1.91	1.27	3.62	3.01	0.54	0.65	0.15	1.16
6	p1.ENSMUST00000131907	46.39	2.91	4.53	4.51	4.10	3.06	2.97	3.62	3.32	3.23	3.78	5.25	2.66	3.66
7	p1.Gm2694	39.41	1.80	3.17	3.99	4.08	4.24	4.78	4.83	4.27	4.32	5.73	6.62	7.92	4.65
8	p1.C230004F18Rik	39.36	-0.05	0.34	0.79	1.55	1.64	1.31	2.44	2.42	1.66	0.97	0.84	3.34	1.44
9	p1.Xist	37.89	0.20	1.59	2.72	3.00	1.01	1.27	1.77	1.35	3.26	2.43	1.44	2.00	1.84
10	p1.ENSMUST00000098868	29.75	1.31	2.98	5.83	4.80	4.35	2.60	2.14	2.19	1.47	0.56	0.01	-0.06	2.35
11	p1.C630043F03Rik	24.13	1.18	2.85	5.45	7.24	5.38	3.46	3.13	2.67	1.59	1.36	1.31	0.62	3.02
12	p1.A730094K22Rik	22.92	0.73	2.16	3.29	4.84	4.88	3.82	6.32	5.45	5.22	5.64	6.92	3.90	4.43
13	p1.ENSMUST00000139356	22.86	3.42	3.40	3.69	2.85	2.73	2.57	2.54	4.05	3.18	2.97	2.44	0.67	2.88
14	p2.Dlg2	20.12	0.04	0.86	2.17	4.32	3.89	3.47	4.89	4.37	3.35	2.63	2.98	4.82	3.15
15	p1.1700020I14Rik	19.71	0.15	0.19	0.32	0.34	0.30	1.05	0.80	0.84	1.01	0.95	0.97	3.40	0.86
16	p9.Dlg2	18.58	-0.20	-0.13	1.87	4.47	6.82	6.07	5.70	6.82	6.92	3.49	2.21	3.00	3.92
17	p3.Peg13	17.56	1.29	2.07	2.64	2.88	2.03	2.11	2.80	3.81	1.97	1.39	1.48	1.80	2.19
18	p2.ENSMUST00000098868	17.47	2.05	5.68	8.21	6.26	4.13	2.11	2.20	2.68	0.87	0.28	-0.05	-0.11	2.86
19	p1.ENSMUST00000128121	17.00	3.82	5.77	5.46	4.38	5.10	3.74	3.19	4.49	3.60	3.53	3.92	2.01	4.08
20	p1.ENSMUST00000133630	16.17	0.54	1.18	2.19	2.81	3.63	3.91	4.08	3.99	3.85	3.77	3.66	1.39	2.92
21	p1.2900079G21Rik	15.16	-0.11	0.50	1.29	1.30	1.52	1.65	2.11	3.04	3.83	3.74	6.85	5.25	2.58
22	p7.Dlg2	14.97	-0.20	-0.13	1.65	4.34	5.71	4.91	5.16	6.53	4.84	2.95	2.64	3.27	3.47
23	p1.ENSMUST00000139835	14.50	0.29	1.14	2.10	2.19	2.28	2.44	2.75	2.75	2.55	3.32	3.07	0.49	2.11
24	p2.Miat	13.84	1.89	3.02	4.32	2.76	3.09	3.00	2.66	3.05	4.80	3.53	4.40	2.34	3.24
25	p3.ENSMUST00000131907	13.50	5.99	6.87	5.38	4.57	4.33	2.77	3.75	4.03	3.02	3.21	3.68	1.48	4.09
26	p1.ENSMUST00000134624	11.74	3.20	2.76	1.86	1.64	0.82	0.86	0.73	0.75	0.32	0.08	-0.10	-0.38	1.04
27	p5.Dlg2	11.48	-0.26	-0.10	2.14	1.83	2.01	1.99	2.37	3.91	4.73	5.06	5.35	7.41	3.04
28	p3.Cct6a	11.32	3.18	3.25	1.95	2.17	1.89	1.75	0.89	1.97	1.02	0.56	-0.21	0.24	1.56
29	p1.ENSMUST00000155166	10.96	1.63	2.70	3.00	3.10	2.50	2.29	2.86	1.90	1.63	1.45	1.23	1.64	2.16
30	p1.2610316D01Rik	10.12	1.34	1.64	2.61	2.59	2.28	2.58	3.20	2.21	1.75	2.10	1.43	0.92	2.05
31	p1.ENSMUST00000123016	9.91	1.46	3.77	6.36	5.25	4.24	3.65	3.18	2.83	2.25	1.86	1.59	0.66	3.09
32	p1.ENSMUST00000151778	9.31	4.48	6.68	6.71	5.83	5.85	4.09	3.30	2.66	2.80	2.26	1.35	0.29	3.86
33	p3.ENSMUST00000098868	8.98	1.30	4.71	7.13	5.60	4.62	2.18	2.54	3.37	1.27	0.45	0.39	0.00	2.80
34	p2.ENSMUST00000131907	8.68	3.24	4.22	3.21	3.56	2.69	2.07	3.20	3.86	3.34	2.43	3.83	3.32	3.25
35	p1.ENSMUST00000170849	7.92	4.73	2.72	1.31	0.78	0.47	0.22	0.09	0.02	-0.10	-0.12	-0.05	-0.16	0.82
36	p1.A230056P14Rik	7.68	2.83	3.23	4.16	3.86	4.33	2.84	3.81	2.54	1.25	0.93	1.37	1.89	2.75
37	p1.ENSMUST00000159155	7.40	-0.11	0.24	0.44	1.39	1.91	2.97	4.21	5.09	4.82	7.70	8.78	2.32	3.31
38	p1.D430036J16Rik	7.23	1.46	1.05	0.48	1.74	2.18	3.63	4.49	4.30	5.04	3.32	1.38	1.88	2.58
39	p1.ENSMUST00000148743	6.81	3.51	1.91	2.05	1.11	1.00	1.16	1.33	0.62	0.66	1.00	0.88	-0.12	1.26
40	p1.ENSMUST00000129089	6.51	5.39	3.18	3.25	2.62	1.46	1.61	1.19	0.63	0.47	-0.11	0.11	-0.38	1.62
41	p1.ENSMUST00000144350	6.44	0.54	2.40	2.40	3.26	3.63	4.45	5.88	6.12	5.35	7.83	7.05	3.85	4.40
42	p1.ENSMUST00000151229	6.37	5.67	3.60	2.79	2.76	1.41	0.73	0.78	0.81	0.21	-0.18	-0.28	-0.42	1.49
43	p1.ENSMUST00000124378	6.11	0.22	1.52	2.57	3.03	3.26	2.92	2.27	1.85	1.74	1.24	0.57	0.52	1.81
44	p3.Meg3	6.00	0.09	-0.15	0.34	0.56	1.98	2.33	1.02	3.26	2.18	0.20	0.42	0.17	1.03
45	p1.2810429I04Rik	5.94	3.21	2.93	2.35	2.94	2.72	3.15	2.34	3.04	2.41	1.60	0.92	1.32	2.41
46	p4.Peg13	5.91	0.86	1.10	1.76	1.97	1.63	1.85	2.20	2.52	2.09	1.37	2.36	3.75	1.96
47	p1.ENSMUST00000129868	5.85	-0.14	0.38	1.96	5.48	6.67	6.27	9.10	9.55	4.98	2.32	1.71	0.15	4.03
48	p1.2610027F03Rik	5.32	7.51	9.22	5.93	2.98	1.07	0.59	0.44	0.06	-0.11	-0.08	-0.11	-0.11	2.28
49	p4.ENSMUST00000098868	5.11	2.14	4.95	7.76	6.47	4.44	2.68	2.28	1.50	1.92	0.58	0.25	0.01	2.91
50	p17.Dlg2	4.29	-0.29	-0.09	0.82	0.60	1.58	2.03	3.56	6.05	5.79	5.76	5.10	6.16	3.09
51	p1.ENSMUST00000098678	4.25	8.06	3.36	2.67	1.78	0.76	0.82	0.03	-0.05	-0.16	-0.19	-0.19	-0.19	1.39
52	p12.Dlg2	4.16	-0.24	-0.23	1.41	1.45	1.68	2.01	1.90	3.58	5.07	4.14	5.40	5.54	2.64
53	p6.Meg3	4.04	-0.02	-0.15	0.17	0.73	1.02	1.28	1.12	2.30	1.35	0.58	0.84	3.71	1.08
54	p1.A330076H08Rik	3.83	-0.06	0.23	0.18	0.50	0.59	0.29	0.57	1.16	1.42	1.39	2.56	11.29	1.68
55	p3.9430053O09Rik	3.74	0.90	2.98	10.67	9.80	6.94	4.13	2.50	1.14	1.63	0.67	0.36	-0.19	3.46
56	p1.ENSMUST00000126472	3.44	4.70	4.20	2.08	1.80	1.76	1.03	1.43	1.01	0.55	0.23	-0.27	0.10	1.55
57	p2.2610027F03Rik	3.29	12.16	9.55	6.12	2.42	0.87	0.46	0.36	0.87	-0.05	-0.12	-0.12	-0.12	2.70
58	p1.ENSMUST00000147294	2.90	3.45	2.31	1.81	1.30	0.84	0.16	-0.02	-0.21	-0.09	-0.19	-0.21	-0.18	0.75
59	p1.ENSMUST00000137258	2.89	12.68	8.08	3.52	0.96	-0.12	0.08	-0.12	-0.12	-0.12	-0.12	-0.12	-0.12	2.04
60	p4.2610203C20Rik	2.81	1.01	1.06	3.47	4.93	4.65	2.99	1.49	5.10	1.82	1.62	2.25	0.55	2.58
61	p1.ENSMUST00000128815	2.79	3.49	4.99	5.67	3.98	2.23	1.68	0.48	0.61	0.29	0.05	0.40	-0.17	1.97
62	p1.ENSMUST00000160099	2.59	0.62	1.01	1.10	1.06	1.39	0.55	1.99	3.14	1.02	0.94	2.32	0.11	1.27
63	p3.2900079G21Rik	2.15	-0.09	-0.09	-0.09	-0.09	0.00	0.03	0.82	0.57	0.97	5.32	9.56	12.06	2.41
64	p1.ENSMUST00000130362	1.98	-0.17	-0.14	-0.09	-0.10	0.15	0.51	0.87	0.50	1.12	2.60	5.83	1.56	1.05
65	p1.ENSMUST00000159595	1.18	-0.09	0.22	0.42	0.53	0.40	0.16	0.45	0.55	0.21	1.49	4.41	9.31	1.50
66	p20.Meg3	1.11	-0.04	-0.04	0.34	0.68	1.32	1.59	0.46	5.96	2.69	0.09	1.44	-0.14	1.20

Robustly expressed lncRNAs in the FANTOM5 cerebellar time course were filtered with the criteria of Z-score ≥3 in at least 1 out of 12 cerebellar timepoints and considered enriched for that corresponding timepoint. This analysis yielded 66 hits that were ranked according to their average expression levels across the 12 cerebellar timepoints, shown here in this table

protein DGCR8 (aka Pasha, in *drosophila*) are required to cleave pri-miRNA to precursor (pre-) miRNA, which are translocated by Exportin-5 (XPO5) into the cytoplasm. Finally, another RNase III enzyme called Dicer dices pre-miRNA to mature miRNA [61]. A complex of the RNA-induced silencing complex (RISC) and mature miRNA bind to the target mRNA, mostly at the 3′-untranslated regions (3′ UTR). miRNAs post-transcriptionally control gene expression and repress protein production by destabilizing the mRNA (mRNA deadenylation/decay) or translational silencing/repression of target mRNAs by interaction with partially mismatched sequences in their 3′ UTR [61]. Also, it has been found that one miRNA

may control hundreds of targets, and one gene (e.g., developmentally relevant genes) may be regulated by several miRNAs [64].

Advances in the study of miRNAs have been immeasurably helped by the use of traditional and newer and more advanced molecular methods [see [65] and Table 2]. To appreciate miRNA activity at the single cell level, which is critical in the analysis of a cellularly complex tissue such as the brain, one needs to use modern anatomical methods. One promising approach that has been recently employed is miRNAscope in situ hybridization [66, 67]. An image of the developing cerebellum is shown in Fig. 1 using this method. In the nervous system of developing mouse and human, the spatiotemporal expression pattern of miRNAs has been demonstrated [9, 68]. An assessment of miRNA expression between different brain regions (prefrontal cortex, hippocampus, and cerebellum) indicates an increase in differentially expressed miRNAs between brain regions over developmental time [69]. From the perspective of this chapter, we are interested in studies that have focused on finding miRNAs enriched in cerebellar development in comparison to other brain subregions [70, 71].

To understand the function of miRNAs, the critical role played by them in development is demonstrated by the Dicer-knockout mice, which exhibits an early embryonic lethality at E7.5 [20]. Giraldez et al. evaluated the role of miRNAs in brain formation by producing maternal-zygotic *dicer* mutants and blocked all miRNAs in zebrafish which resulted in defects in brain morphogenesis, somitogenesis, and heart development [72]. During brain development, the subdivision of the vertebrate CNS appears under the control of miRNAs. The organizing activity and progenitor state of the midbrain–hindbrain boundary (MHB) are co-regulated by a single miRNA, miR-9, during late embryonic development and targets several components of Fgf signaling pathway [73]. In contrast, miR-10 is as a caudalizing factor, and it

Table 2 Experimental techniques to detect miRNAs

Method	Comments	References
Northern blotting	Traditional technology, used for detecting mature and precursor miRNAs	[106, 107]
Real-time qPCR	Gold standard technique, highly sensitive	[108–110]
Microarray	Rapid but cost very high, hard to detect too short or low miRNAs	[111, 112]
miRNA sequencing	Genome-wide profiling and analysis of known, as well as novel, miRNA variants	[113, 114]
Nanoparticles (gold, silver, magnetic, and quantum dots)	Pros: Powerful versatility of cellular transfection, excellent photostability and low immunogenicity Cons: Inherent cytotoxicity and self-aggregation inside living cells	[115–118]
Nucleic acid amplification techniques	Detect low content of miRNAs	[119]
miRNAscope in situ hybridization (ISH) assay	Highly sensitive, spatial assessment of miRNAs on tissue sections	[66, 67]
miRNA luciferase reporter assay	Gold standard of the in vitro assays to validate miRNAs and their specific target	[120–122]

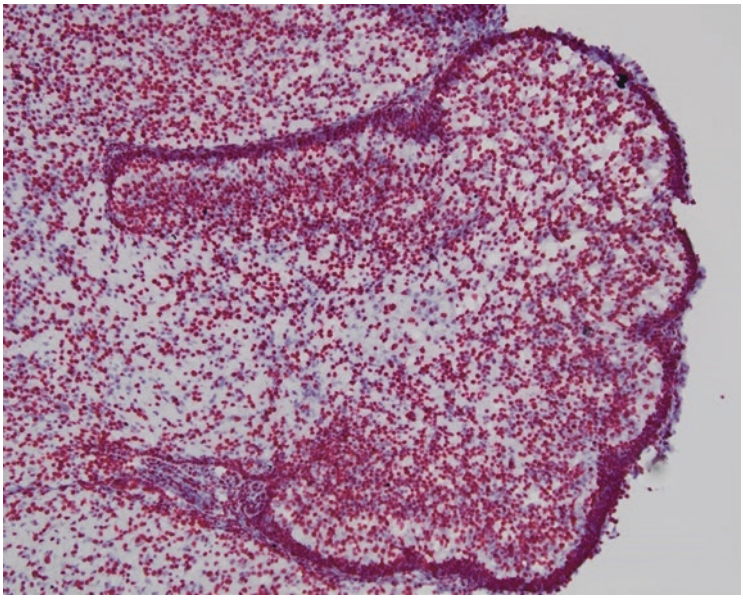

Fig. 1 miRNAscope ISH assay. Using miRNAscope™ HD (RED) Assay a probe to U6miRNA (SR-RNU6-S1, Cat# 727871, Advanced Cell Diagnostics) was used on P0 (postnatal day 0) mouse cerebellum (C57BL6/J). As expected, the U6miRNA is abundantly expressed in almost all nuclei

is expressed in hindbrain and spinal cord while it is absent in rostral regions [74]. miR-10 has been shown to downregulate key midbrain markers as Otx2 and to upregulate hindbrain markers caudal to mid-hindbrain boundary (MHB) as Gbx2 in human in neural progenitor cells (NPCs) [75]. The expression pattern of miR-9 and miR-10 at the isthmic organizing center (located at the MHB) reminds one of the expression patterns of Otx2 and Gbx2. These two transcription factors meet to position MHB and direct the development of the midbrain and anterior hindbrain [76, 77]; suggesting as noted above that miR-9 and miR-10 are similarly involved.

The requirement of miRNA machinery in early cerebellar development in the mouse has been explored using the conditional ablation of DICER targeted to the cerebellar anlage through the use of Wnt1-Cre. This conditional deletion of DICER at the midbrain-hindbrain boundary around E12.5 results in the elimination of several miRNAs in the midbrain and rostral hindbrain and dramatic malformation of the tectum and cerebellum [78]. The most dramatically reduced miRNAs were miR-9, miR-124, and miR-280 in the midbrain and rostral hindbrain.

The expression profiling of miRNAs in isolated cerebellar cells has provided a view as to changes in miRNA populations over development. Pieczora et al. profiled miRNAs expressed in isolated rat Purkinje cells using laser microdissection at postnatal development and found differentially expressed miRNAs which show up- and down-regulation at postnatal day (P) 9 to P30 [79]. This may be indicative of the developmental events which largely occur during the first three postnatal weeks of life, when Purkinje cells develop dendrites and establish synaptic connections [80].

In ongoing research from our lab, miRNA expression has been profiled at early postnatal days in isolated cerebellar granule cells. During these times, granule cells engage in massive cell proliferation, migration and differentiation/maturation. Our primary findings indicate a dramatic increase in differentially expressed miRNAs (up- and downregulated) between early and later postnatal timepoints which align with a dynamic role for miRNAs during development.

The importance of miRNAs in the development of three principal cerebellar cells, Purkinje cells, granule cells and astroglia, have been demonstrated. For Purkinje cells, work by Schaefer et al. using a *dicer* conditional null driven by a Purkinje cell-specific Pcp2-cre resulted in Purkinje cell loss and development of ataxia [81]. For granule cells, Ferretti et al. identified a role for miRNAs in Sonic Hedgehog (SHH) signalling in cultured granule cell precursors [82]. To turn on SHH signaling, SHH binds to its receptor Patched 1 (PTCH1) which results in PTCH1-mediated inhibition of SMO (Smoothened; a G-coupled transmembrane receptor) to be lifted, allowing for constitutive SMO activity and associated downstream signaling (including Gli; Zinc Finger family protein, glioma-associated oncogene) [83]. Ferretti et al. demonstrated that miR-125b, miR-324-5p and miR-326 antagonize this major mitogenic stimulus to granule cells, Sonic Hedgehog, by targeting Smo and Gli to promote cerebellar granule cell differentiation [82]. Furthermore, a study by Ma et al. in granule cell precursors has shown that miR-9, under N-myc control, is implicated in granule cell proliferation [84]. Finally for astroglia, Tao et al. and Kuang et al. targeted Dicer deletion in astrocytes using two different conditional Gfap-cre lines of mice [85, 86]. They found an early and severe defect in Bergmann glia that is followed quickly by major deficits in cerebellar morphogenesis [85] or at later postnatal times widespread granule cell loss with a degeneration of Purkinje cell dendrites [86].

miRNAs Expression in Cerebellar-Related Disorders

The deregulation of miRNA expression has been reported in a wide range of cerebellar-related disorders, such as medulloblastoma, a variety of ataxic syndromes, autism spectrum disorder, and Rett syndrome [87–91].

The most extensively studied aspect of miRNAs in cerebellum is the association of miRNA expression and medulloblastoma, the most common malignant childhood brain tumours that arise from cerebellum and hindbrain [88–90]. Expression profiling of miRNA in human reveals that miRNAs are differentially expressed in medulloblastoma compared to healthy tissue, indicating a potential involvement of miRNAs in the etiology of disease. In addition, miRNA profiling has identified distinct miRNA signatures associated with each molecular subtype of medulloblastoma, alongside unique mRNA markers that would predict clinical outcome [90, 92]. Hence, miRNA profiling is used for molecular classification of medulloblastoma to inform risk stratification and therapeutic intervention [93–98].

Spinocerebellar ataxia type 3 (SCA3) is caused by a polyglutamine expansion in the deubiquitinating enzyme Ataxin-3, and this gene targets miRNAs mir-9, mir-181a, and mir-494. In SCA3, these miRNAs are dysregulated. To validate a miRNA approach to therapeutic intervention targeting the 3′UTR of human ATXN3, an artificial miRNA was injected into the region of the cerebellar nuclei. This treatment reduced ATXN3 expression, which did not alter the levels of E4B, a well-known Ataxin-3 interacting protein, nor did it lead to gross changes in the morphology of cerebellar nuclei neurons [99, 100]. In SCA7, which is caused by a CAG repeat expansion in the ATXN7 gene-coding region, the altered expression of circulating miRNAs, hsa-let-7a-5p, hsa-let7e-5p, hsa-miR-18a-5p, and hsa-miR-30b-5p, were explored. From a gene ontological approach, the target genes of these miRNAs are enriched in the biological categories of Fas-mediated cell-death, heparan sulphate biosynthesis, and soluble-N-ethylmaleimide-sensitive factor activating protein receptor pathways; all involved in neurological function [101]. In Fragile X-Associated Tremor/Ataxia Syndrome, caused by an expanded CGG repeat in the FMR1 gene, there is a decrease in miRNA in brain due to a preferential binding and sequestration of DGCR8 to the CGG repeat. This appears to be pathogenic as when DGCR8 is overexpressed in mouse cortical cells or neuronal cells lines with the expanded repeat the phenotypes of dendritic pathology and cell death are reversed [102].

A role of miRNAs in autism spectrum disorder has been suggested in several studies. In the investigation of post-mortem cerebellar cortex of ASD patients, aberrant expression of nine miRNAs targeting two well-known ASD genes, neurexin (NRX1) and Shank3 mRNA, was identified [75]. Furthermore, lymphoblastoid cell lines derived from ASD patients were studied to identify the differentially expressed miRNAs and many of differentiated expressed miRNAs were found with neurologically relevant target genes [103, 104].

In Rett syndrome, the story of miRNAs is different and MeCP2 (methyl CpG binding domain protein-2, a silencing factor at methylated DNA sequences) which interacts with chromosomal miRNAs in brain and all MeCp2-interacting miRNA target genes are inhibited which result in modulation of gene expression [105]. In this study, it has been revealed that the inhibition of these target genes caused the dysregulation of neurological pathways such as: ligand gated ion channels and GABA A receptor activation was inhibited, and carbohydrate metabolism and L1CAM interaction pathways were induced.

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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 proper behavior.

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

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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 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 descending motor systems of 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. Moreover, an emerging line of investigation suggests a role for the cerebellum in reward processing. In all cases, 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 or compensation 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, that are 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 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 lobule (Pml), copula pyramidis (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

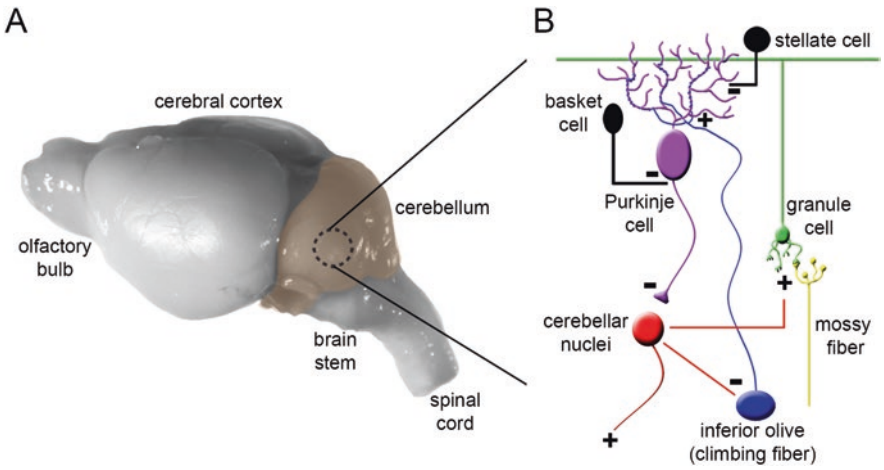

Fig. 1 Architecture of the cerebellar circuit. (**a**) Mouse brain shown from a lateral view with the cerebellum highlighted in color. (**b**) The basic cerebellar circuit comprises Purkinje cells, granule cells, stellate and basket cell interneurons, and the cerebellar nuclei. Afferent information is 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. (Modified with permission from Ref. [92])

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 [13]. These nuclei include the basilar pontine nuclei relaying input from 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 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 extrinsically influence local activity [14, 15]. Purkinje cell axons are the sole output of 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 main cerebellar efferent pathway to the rest of the brain and spinal cord; however, a subset of Purkinje cells projects directly to vestibular nuclei [16]. 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 [17]. The topographic map of zebrinII expression in mice has been detailed extensively [18–20]. However, zebrinII is conserved, and its

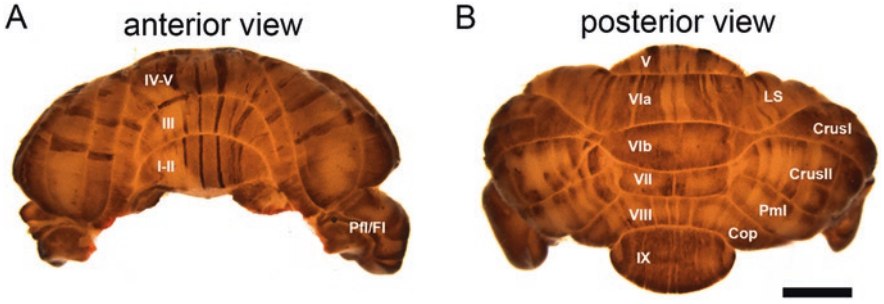

Fig. 2 ZebrinII zones (stripes) in the mouse cerebellum. (**a**, **b**) Wholemount immunohistochemical staining of the mouse cerebellum with zebrinII reveals the intricate patterning of the cerebellar cortex into parasagittal zones. Roman numerals identify the lobules of the vermis. *Pfl* paraflocculus, *Fl* flocculus, *LS* lobulus simplex, *Pml* paramedian lobule, *Cop* copula pyramidis. Scale bar = 2 mm. (Modified with permission from Ref. [92])

general pattern of expression is identical across different taxa [21–27]. 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 -negative zones that are symmetrically distributed across the midline. More than 40 molecular markers of zones have been identified [28], 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 [29] (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, 30]. 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 [29]. 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 [31, 32] or the narrower functional microzones [33]. Climbing fibers originating from a specific subnucleus of the inferior olive typically terminate in one or two of these longitudinal zones [34, 35], and mossy fibers from specific sources branch to terminate in multiple longitudinal zones [36–39]. Zones are also distinct in their topographically defined Purkinje cell output to specific subnuclei of their three target cerebellar nuclei: fastigial (medial), interposed (intermediate; = globose and emboliform in primates), and dentate (lateral), each of which has a unique efferent pathway to the rest of the brain and spinal cord [30, 40, 41], including projections back to the inferior olive to form a patterned cortico-nucleo-olivary tripartite loop [42, 43]. Together, units of topographically organized cerebellar afferents, their target Purkinje cell zones, and Purkinje cell efferent projections to the cerebellar nuclei comprise cerebellar “modules,” the basic functional circuit of the cerebellum [44]. Retrograde transsynaptic tracing shows that individual muscle groups are linked to specific Purkinje cell zones [45]. Functional mapping of the cerebellar circuit using imaging and electrophysiology also exhibits topography consistent with the zonal plan [46–49]. 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 [46, 50, 51]. Due to the relatively uniform cytoarchitecture of the

cerebellum, it has been thought that these topographical differences in function arise due to 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 [52]. 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 [53]. During this complex coordination, there are many opportunities for insults to cause disorders with devastating consequences for motor and even non-motor behavior.

Development of the Cerebellar Circuit

Due to the cerebellum's well-understood circuitry and potential roles in developmental and adult-onset diseases, it is an important model for understanding normal and abnormal brain circuit map formation [53]. Positional cues must be present to set up the patterns of specific lobules in the anteroposterior axis and zones in 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, at around 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 [53]. 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 substantially and fold as they subdivide into the conserved stereotyped lobules, and this process (lobulation) is close to complete by postnatal day 14 (P14) in mice, although growth and patterning continue until around P21. Genetic cues allowing for the precision and reproducibility of lobulation 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 [54] under the control of Purkinje cell-derived sonic hedgehog (Shh) signals [55, 56] and the function of *Engrailed* homeobox genes (*En1/2*) [57, 58]. 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

[59–61], including cell adhesion and guidance molecules [62, 63]. Purkinje cells are critical not only for shaping morphogenesis but also for guiding topographic 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 [64]. Afferents arrive in the cerebellum spanning mid-embryonic and postnatal development [65] 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 [53]. Retrograde tracing in fixed embryonic rat tissue shows mossy fibers from the vestibular ganglion arriving in the cerebellum by E13, and those from the vestibular nuclei and spinal cord arriving at E15 [65]. Climbing fibers arrive at ~E17, followed by mossy fibers from the lateral reticular nucleus and pontine nuclei at P0 [65]. In mice, spinocerebellar and vestibular mossy fibers arrive at E13/14 [66], climbing fibers arrive at E14/15 [67], and the remaining mossy fibers arrive during late embryonic and postnatal development [53]. Climbing fiber afferents exhibit rudimentary parasagittal stripes by E15/16 in mice [67], soon after Purkinje cell clusters initially express transient parasagittal molecular markers such as *En1/2* [60]. Climbing fiber termination patterns and Purkinje cell zones correspond topographically by E17 [68]. Though mossy fibers synapse on granule cells in the adult cerebellum, they form transient contacts with Purkinje cells during embryonic and early postnatal development that may be critical for the segregation of spinocerebellar afferents into parasagittal zones [31, 69–72]. Unlike climbing fibers, mossy fibers do not exhibit clear-cut zones until after birth [73]. 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 [74]. 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 [75] (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 [75]. The development of Purkinje cell electrophysiology, morphology, and associated sensorimotor behaviors additionally relies upon the unique zonal patterning of the cerebellum as it was discovered that Purkinje cells of the posterior cerebellum (ZebrinII-positive lobule X) reach their adult stage prior to those of the anterior cerebellum (ZebrinII-negative lobule III), corresponding to a decrease in anterior-dependent eyeblink conditioning but faster nodular-dependent compensatory eye movement adaptation [76]. Neural activity, mediated by spontaneous activity and sensory experience, likely also intersects with genetic programs to properly assemble the cerebellum and its circuits [77]. In fact, the zonal arrangements of both inhibitory projections from basket cells onto Purkinje cells and excitatory mossy fibers onto granule cells require Purkinje cell neurotransmission [78, 79]. Similarly, the proper maturation of the anatomical and electrophysiological properties of Purkinje cells relies upon the neurogenesis of excitatory

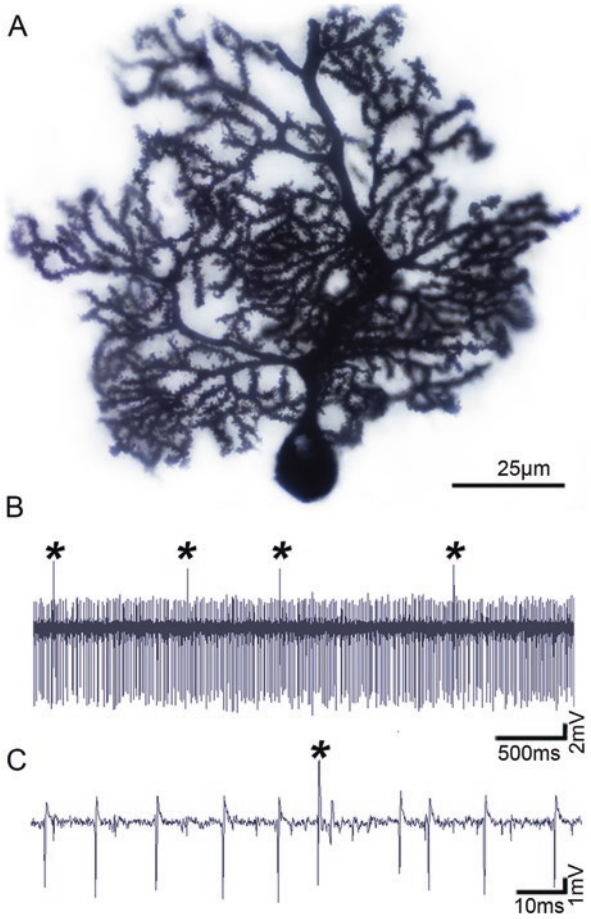

Fig. 3 Purkinje cells fire simple spikes and complex spikes. (**a**) Purkinje cell labeled using the classic Golgi-Cox staining method, demonstrating the elaborate morphology and dendritic branching of the Purkinje cell. (**b**) Extracellular single-unit recording from a Purkinje cell of an adult mouse 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 (asterisks). (**c**) Higher power image of the Purkinje cell recording shown in panel (**b**) with individual spike waveforms visible. (Modified with permission from Ref. [92])

granule cells [80]. 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, the *Engrailed* homeobox transcription factor family is critical for establishing the organization of the cerebellar circuit, and *En1/2* mutants exhibit altered formation of lobules and parasagittal Purkinje cell gene expression [58, 81–84]. Furthermore, adult patterns of mossy fiber afferents in distinct lobules and parasagittal zones are sensitive to *En1/2*

deletions [71]. In addition, components of the endocannabinoid signaling system such as cannabinoid receptor 1 (CB1) have been recently demonstrated to be expressed in a developmentally dynamic, region and cell type-specific pattern in E17.5-P12 mouse cerebella, and that conditional knockout of CB1 in mice leads to selective anatomic alterations of the anterior cerebellar vermis with corresponding motor impairments [85]. 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 [69]. The *dreher* mutation causes cell fate changes of cerebellar progenitors, and anteroposterior and parasagittal patterns are distorted but present, despite external morphological phenotypes [86]. 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 [87]. *Scrambler* mutant mice are able to attain and maintain Purkinje cell zones and topographical circuits despite the abnormal placement of 95% of Purkinje cells due to severe ectopia [88]. The *reeler* mutation causes the cerebellum to contain a "single lobule" composed of a hypogranular cortex and a central mass of Purkinje cell clusters mixed with the 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 [89, 90]. These mouse models of motor dysfunction, which have cerebellar abnormalities due to structural and circuit defects, have therefore 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 diseases that involve the cerebellum.

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 incoordination of the limbs, impaired balance, gait disturbance, and diminished fine motor control [91]. Cerebellar ataxia is the most common form of ataxia, and there are currently over 60 identified forms of inherited cerebellar ataxia [92, 93]. 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 [94]. 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. Like other cerebellar ataxias, 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 [95]. 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* [96]. The polyglutamine ataxin-1 protein product is widely expressed in the brain, and its polyglutamine expansion further stabilizes ataxin-1, facilitating its toxic accumulation in the nucleus of affected neurons [97, 98]. Among these neurons, the Purkinje cells of the cerebellum are a primary target [99] as polyglutamine ataxin-1 remains uniquely soluble in Purkinje cells, allowing it to enter the nucleus and disrupt the function of multiple protein complexes [100]. 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 [101]. Neuroimaging of late-stage SCA1 patients reveals gross atrophy of the cerebellum primarily due to the degeneration of Purkinje cells [95, 99, 102]. SCA1 patients also typically exhibit atrophy of the dentate cerebellar nuclei, pons, inferior olive, and other brain stem nuclei as the disease progresses [99]. Thus, degeneration eventually impacts both the cerebellar afferent and the efferent pathways. Postmortem examination of cerebellar tissue from SCA1 patients shows morphological abnormalities of the remaining Purkinje cells in addition to Purkinje cell loss [102, 103]. The generation of mutant SCA1 transgenic mice has been critical in furthering our understanding of SCA1 progression [104–106]. For instance, 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 [107, 108]. These functional changes correspond with abnormalities in the structural development of Purkinje cell inputs. Due to the hyperproliferation of cerebellar stem cells and their preferential differentiation into GABAergic inhibitory interneurons during the first three postnatal weeks, the number of inhibitory basket cell synapses is markedly increased [109] while climbing fiber innervation is decreased by 5 weeks of age when symptoms first manifest [110]. This early shift in inhibitory/excitatory balance on the Purkinje cell may underlie their vulnerability to SCA1 pathogenesis and abnormal function

during adulthood [109]. 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 [111, 112]. Impaired performance on motor tasks in SCA1 mutant mice appears subsequently but before Purkinje cell morphological changes [107], 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 [104, 107]. 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 [104, 106, 107]. It is not until the later stages of disease progression that Purkinje cell loss is detected [104, 106, 107]. 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 [101]. 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. Interestingly, recent work suggests a region-specific vulnerability to SCA1 pathology within the cerebellum in which only specific regions are altered while others are left functionally and morphologically intact [113]. In the ATXN1[82Q] mouse model of SCA1, which expresses human polyQ-expanded ATXN1 specifically in Purkinje cells, the structure and function of the flocculonodular lobes and crus1 were unperturbed while those of other cerebellar lobules were impaired [113]. This region-specific vulnerability to degeneration may be mediated by local changes in sphingolipid metabolism as it was demonstrated that these patterned areas of Purkinje cell neurodegeneration in ATXN1[82Q]/+ mice correspond to regional differences in sphingolipid metabolism and that partial restoration of these changes via genetic mutation leads to a neuroprotective effect on Purkinje cells [114]. Toward developing treatments for SCA1, several groups are currently focusing on reducing the overaccumulation of polyglutamine ataxin-1 through a decrease in S776 phosphorylation, which reduces the stability of ataxin-1 [115, 116]. The authors found that a reduction in the levels of mutant ataxin-1 through decreased S776 phosphorylation improves motor coordination, neuromuscular respiratory dysfunction, and the life span of SCA1 mutant mice, but that this treatment demonstrates only an attenuated rescue in mice with disrupted S776 phosphorylation in both the mutant ataxin-1 allele and wild-type allele [117]. This suggests a brain region-specific disease mechanism for SCA1 and implies a neuroprotective effect for wild-type ataxin-1 [117]. How exactly these different alleles of ataxin-1 contribute to SCA1 disease pathogenesis and normal function, particularly in the cerebellum where the effects are most evident, remains to be fully resolved.

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 [118, 119]. The mutated polyglutamine P/Q-type calcium channels are widely expressed in the brain but become toxic primarily to Purkinje cells [120], where they are highly expressed in the plasma membrane [121]. 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 [101]. SCA6 patients experience slowly progressive ataxia of the limbs and gait in addition to dysarthria and nystagmus [118, 122], and neuroimaging reveals cerebellar atrophy [122]. Neurodegeneration in SCA6 occurs mostly in Purkinje cells, but death of neurons in the dentate cerebellar nuclei and inferior olive is also observed [119, 123, 124]. Postmortem examination of cerebellar tissue from SCA6 patients shows morphological abnormalities of the remaining Purkinje cells in addition to the loss of Purkinje cells [120]. In transgenic mouse models of SCA6, the onset of ataxia occurs before morphological changes or loss of Purkinje cells [125]. Electrophysiological examination reveals that Purkinje cells exhibit reduced firing rates and rhythmicity at ages coinciding with the onset of ataxia [126] and at later disease stages [127]. Though the polyglutamine mutation occurs in an ion channel that regulates the firing patterns of Purkinje cells in adult mice [128], 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 [127, 129, 130]. Although SCA6 symptoms manifest in midlife, P/Q channels are expressed soon after birth [131] and are involved in synapse elimination of climbing fiber innervation onto Purkinje cells during development [74, 132, 133]. 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 [134]. These alterations disappear once the mice reach weanling age when the circuit has largely developed [53], and cellular and synaptic functions of Purkinje cells return to normal [134]. 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 [108]. 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 [134]. In addition to SCA1 and SCA6, a prolonged period of Purkinje cell dysfunction prior to neuronal loss has also emerged as a common feature in other models of ataxia. Purkinje cells in a genetic mouse model of spinocerebellar ataxia type 3 (SCA3) exhibit abnormal

intrinsic activity and motor symptoms prior to neurodegeneration [135]. In a novel mouse model of ataxia-telangiectasia characterized by progressively severe ataxia and atrophy of the cerebellar molecular layer, Purkinje cells display significant alterations in firing properties and morphology preceding cerebellar atrophy and the onset of behavioral deficits [136]. Similarly, cerebellar developmental deficits (loss of GABAergic connectivity, disrupted climbing fiber development, increased parallel fiber-Purkinje cell connectivity) and motor deficits in a mouse model of spinocerebellar ataxia 23 (SCA23) occur before Purkinje cell loss [137]. Purkinje cell-specific deletion of Ataxia-Telangiectasia and Rad3-related (ATR) protein, the key gene mutated in ataxia-telangiectasia, results in striking locomotor dysfunction and abnormal intrinsic firing activity despite retaining normal structure and morphology of the cerebellum [138]. These early manifestations of ataxias could be effective targets for therapy as the circuits may retain enough functional and structural integrity to be rescued before the cells die or symptoms worsen [107, 126, 135].

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

The carbonic anhydrase 8 gene (*Car8*) is abundantly expressed in Purkinje cells [139, 140]. Lower levels of expression can 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 [141] and is expressed beginning in embryonic development continuing into adulthood [142, 143]. 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 [139]. In humans, mutations in the homologous gene (*CA8*) also cause ataxia [144]. Unlike in the SCAs, Purkinje cells do not exhibit overt degeneration, and the cerebellum does not show gross anatomical defects [139, 140]. However, *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 [145]. The mutation also impairs the topography of cerebellar circuit formation 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 [140] (Fig. 4). Furthermore, electrophysiological examination of mutant mice reveals that the developing Purkinje cells exhibit abnormal firing frequency and patterns [140, 145], but Purkinje cells still do not degenerate and die even as ataxia worsens [140]. 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. In fact, one study found that targeting 13 Hz deep brain stimulation in the interposed cerebellar nucleus of *Car8^{wdl}* mice results in short-term and long-term motor improvements, and that this treatment requires Purkinje cell neurotransmission to be effective [146]. Interestingly, the CAR8 protein is a binding partner for

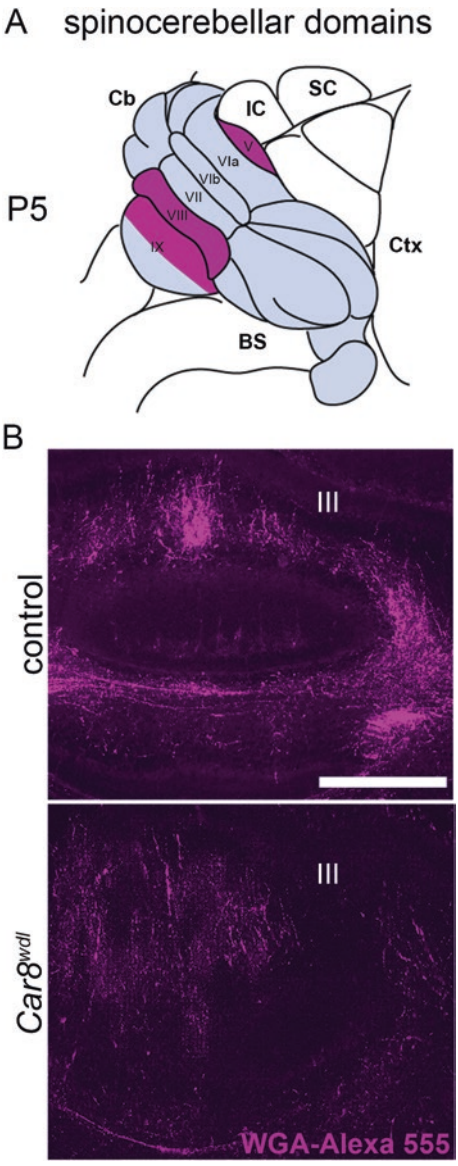

Fig. 4 The termination pattern of spinocerebellar mossy fibers is altered in *Car8*^{wdl} mice. (**a**) Schematic of the postnatal day 5 (P5) mouse cerebellum from a lateral view with the cerebellum highlighted in blue and the primary target domains of spinocerebellar mossy fiber projections highlighted in magenta. Roman numerals identify the lobules of the vermis. Note that the anterior-most 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 Ref. [140])

inositol triphosphate receptor type 1 (IP3R1) [139, 141], an intracellular calcium release channel that is mutated in SCA15. As *IP3R1* is also one of the genes down-regulated in SCA1 mice preceding onset of ataxia or morphological changes [111, 112], impaired calcium homeostasis in Purkinje cells may mediate a central mechanism of pathogenesis common to many types of ataxia that manifest with or without neurodegeneration.

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 [79]. Under control of the cell type-specific promoter *L7* (also called Pcp2 or Purkinje cell-specific protein 2), Cre recombinase excises the *floxed* vesicular GABA transporter gene (*Vgat*) that encodes the transporter for loading neurotransmitter into synaptic vesicles [79]. This eliminates the ability of Purkinje cells, the sole output of cerebellar cortex, to communicate with the cerebellar nuclei, the predominant 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 incoordination, 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 [79]. Although the basic circuit map is intact, the normally sharp boundaries of zones are compromised [79]. Purkinje cells of *L7^{Cre};Vgat$^{flox/flox}$* mice exhibit abnormal electrophysiological activity, but their output is not signaled downstream in this model [79]. However, loss of Purkinje cell signaling causes the cerebellar nuclei to fire abnormally, impacting the ultimate output of the cerebellum. The abnormalities in Purkinje cell activity may be partially attributed to the anatomical rearrangement of its inputs, which typically rely on Purkinje cell neurotransmission for proper patterning [78]. For example, the patterning of both excitatory mossy fibers onto granule cells [79] and inhibitory projections from basket cells onto Purkinje cells are both altered in *L7^{Cre};Vgat$^{flox/flox}$* mice [78]. 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 [147–149]. This idea is supported by evidence of extensive afferents and efferents interconnecting the cerebellum with prefrontal and parietal cortex [40, 150, 151]. Lesioning studies also suggest that cerebellar damage can lead to a variety of non-motor behavioral deficits [149, 152, 153]. However, the extent of the cerebellum's role in cognitive function remains unclear and is a topic of lively debate [154–157]. 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 responses are developed through repeated practice [151, 158]. 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 [159]. Signals from the 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 [53]. 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 [53]. Together, these cerebro-cerebellar connections form closed loops in which regions of cerebellar cortex projecting to a given area of cerebral cortex in turn receive input originating in those same areas of cerebral cortex [40]. Each of these regions is involved in specific functions, forming a topographical map across the cerebellar cortex, cerebellar nuclei, thalamus, and cerebral cortex [30, 40, 41]. Functional neuroimaging links different cognitive and motor behaviors to activity in specific cerebro-cerebellar closed loops [160], and focal cerebellar damage can cause different motor or non-motor deficits in a location-dependent manner [149, 153]. This anatomical and functional segregation of cerebro-cerebellar connections might respect the modular architecture of the cerebellum [44]. Anatomical and functional abnormalities in the cerebellar circuit have been implicated in several non-motor neurodevelopmental disorders [161] and may play a particularly important role during sensitive periods of development [162]. Clinical studies have also noted increased cognitive deficits in children who suffer cerebellar damage during posterior fossa tumor resection [163]. How the cerebellum interacts with cerebral cortex during development remains poorly understood. Some non-motor diseases linked to cerebellar development include autism spectrum disorder [162, 164, 165] and dyslexia [166, 167]. The cerebellum could also be involved in schizophrenia [168, 169]. 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 [164, 170, 171]. The *EN2* gene is necessary for establishing the structure and circuit organization of the cerebellum during

development [53], and *EN2* mutations are linked to autism susceptibility in humans [172–174]. Loss-of-function mutations and transgenic misexpression of *En2* in mice cause autism-like behaviors [175, 176]. 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 [58, 82–84]. 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 [177, 178] and *CA8* mutations [144] cause cognitive deficits in addition to ataxia. It could be that the Purkinje cell and its associated microcircuits underlie both motor and non-motor problems. This would suggest that the basic operational properties of a Purkinje cell could be tuned to different behaviors [179]. Future experimental work will reveal whether this is the case, and indeed evidence is mounting for how Purkinje cells might functionally interact with the hippocampus and prefrontal cortex during non-motor behavior [180].

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Conflicts of Interest We have nothing to disclose.

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A Comparative View of Cerebellar Morphology and Diversity in Fishes

Benjamin W. Lindsey

Abstract Fish represent the most diverse vertebrate class. Through evolutionary time and habitat adaptations, bony and cartilaginous fishes have taken up nearly every aquatic environment of the globe imaginable. These factors have uniquely shaped brain growth, morphology, and even the appearance of functional specializations. The mature cerebellum of different fish lineages is largely reflective of these pressures, providing an unprecedented opportunity to study how this structure has become specialized and has diverged in morphology compared with other vertebrate groups. At a functional level, accumulating evidence points toward a multifaceted role of the fish cerebellum, involved in diverse processes such as movement, cognition and emotion, and sensory-motor learning. While early cerebellar development appears to be largely conserved across vertebrates, including fish, numerous features set these species apart, making them fascinating models to better understand neurodevelopment and environmental pressures. The goal of this chapter is to provide an overview of the distinctive features that characterize the cerebellar architecture of major fish lineages.

Keywords Cerebellum · Cerebelloid structures · Bony fish · Cartilaginous fish · Morphology · Neurodevelopment · Neurogenesis · Comparative

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Introduction

Fish represent the most diverse vertebrate class [39], having adapted to nearly every aquatic habitat possible and representing the most phylogenetically ancient vertebrate lineages. This makes extant fish species tremendously attractive to study the evolution of brain development and specialization. In many cases, lifestyle specializations are reflected by changes in the morphology of brain structures over ontogeny that are coupled with the environment and functional needs [40]. This is equally true of the fish cerebellum that has maintained a basic organizational plan similar to that of their land relatives [34], but this has also been modified by environmental factors as a consequence of major fish radiations. Representing basal vertebrates [7, 67], Chondrichthyes (sharks and rays) and Osteichthyes (bony fish), which additionally include Sarcopterygians (lobe-finned fish) and Actinopterygians (ray-finned fish), serve as extremely valuable models to study the evolution of cerebellar development (*see* Fig. 1). Comparative neuroanatomical investigations of the cerebellum between different major fish lineages as well as tetrapods can provide a rich understanding of the evolution of the cerebellar structure–function relationship.

The cerebellum of fishes displays by far the most structural variation compared to any other vertebrate class [44]. With this in mind, the overarching goal of this chapter is to highlight how such diversity in structure has arisen and how the cerebellar architecture of fishes has over time deviated or been to some extent remodeled, from the fundamental vertebrate cerebellar organizational plan. This review is by no way meant to be exhaustive but rather to provide an overview, as several excellent in-depth reviews investigating cerebellar development and diversity in fishes have been previously published [34, 57, 66, 67, 72, 74, 93]. Across major groups of fishes, most of our current-day knowledge arises from in-depth studies of

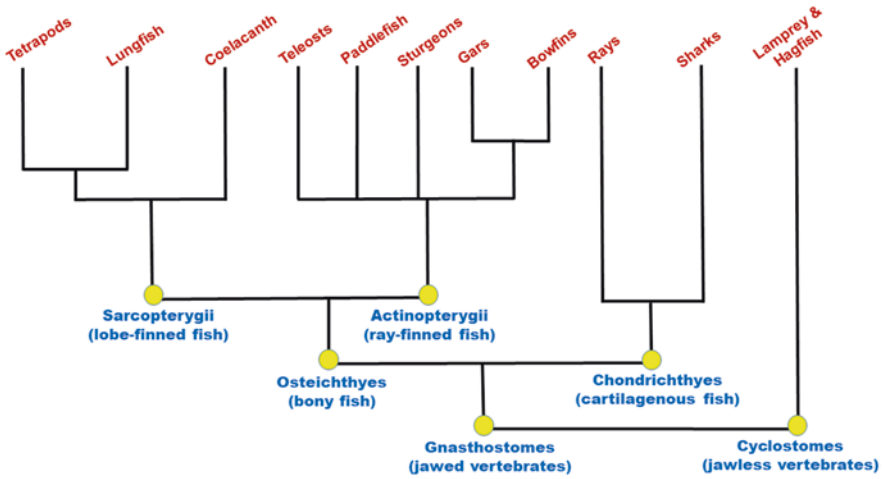

Fig. 1 Cladogram showing the relationship between major groups of fishes

the teleost cerebellum—considered to represent the foundational cytoarchitectural form of the fish cerebellum [67]. This neuroanatomical blueprint provides us with a morphological reference map as we consider deviations from this plan as a result of divergent radiations or habitat and behavioral specializations.

In an attempt to put into perspective the extensive literature on the fish cerebellum that has spanned over a century, this chapter is organized into three sections. *First*, I describe the common cerebellar developmental plan observed from studies in teleosts and how the morphology of the mature cerebellum compares to that of other vertebrates. *Second*, I will provide direct examples of how the cerebellum has diversified across major fish lineages and the role of the environment in shaping the mature cerebellum. *Finally*, this chapter comes to a close by briefly discussing the lifelong neurogenesis present in the cerebellum of many fish species, opening the door to exciting opportunities to explore the function of these cells using a combination of traditional and modern-day experimental approaches. It is my hope that by the end of this chapter readers gain a more robust comparative understanding of the cerebellar architecture of fish from development to adult.

Development of the Fish Cerebellum and Its Structural Organization

Similar to other vertebrates, fish share a highly conserved cerebellar developmental plan. Pioneering comparative work by R. Nieuwenhuys [67] has shown that the origins of the vertebrate cerebellum are commonly derived from the rostral rhombencephalon where two bilaterally symmetrical anlages (i.e., also known as embryonic domains or territories) are dorsally situated. As embryonic development proceeds, these domains fuse in the midline plane. Meanwhile, the rhomboid fossa widens and the angle between the cerebellar territories and two sides increases eventually leading to the fused halves of the cerebellar primordium producing a transverse-oriented plate-like structure [67]. In rodent models, a similar process has been described whereby the rostrocaudal axis of the cerebellar anlage undergoes a 90° rotation to then become the mediolateral axis [76]. The formation of this plate and subsequent growth are driven by early waves of neurogenesis that give rise to the different types of cerebellar neurons that will populate the mature structure. A tightly regulated sequence of progenitor activity arising from two distinct germinal zones, the ventricular zone and upper rhombic lip, are responsible for producing final neuronal subtypes (reviewed in Carletti and Rossi [13], Kaslin and Brand [34]). Comparative work across model vertebrates has revealed, however, slight deviations in the expansion of the cerebellum and the migration pattern of early progenitors [12].

Molecular and genetic characterization of cerebellar development in vertebrates shows that the initial phase of midbrain and cerebellar development commonly depends on the isthmic organizer situated at the midbrain–hindbrain boundary.

While the finer nuances of this process are beyond the scope of this chapter, excellent reviews have shown that a complex cascade of molecularly driven temporospatial events controls early cerebellar specification, including key pathways such as Fibroblast Growth Factor and Wnt, along with a host of transcriptional factors; importantly *otx* and *gbx* [34, 54, 86, 88, 91]. These in turn lead to the establishment of distinct cerebellar territories and provide cues to specify the later cytoarchitecture of the mature cerebellum. To date, this process in fishes has been best described in the zebrafish (*Danio rerio*) model, owing to the high amenability of transparent embryos and rapid development to larval stages ([33, 37, 91]; reviewed in [34]).

Of all jawed vertebrates, fish, along with birds and mammals, maintain the largest adult cerebelli and display the most pronounced structural diversity [68]. With the exception of Cyclostomes (also known as Agnathans; hagfish and lamprey), the cerebellum is characterized by a major lobe, the centrally located corpus cerebelli, and two bilateral lobes, known as the auricles (flocculus in tetrapods; also known as the vestibulocerebellum; [2]). The auricle is considered a specialized domain of the corpus primarily receiving vestibular input [34]. In fish, it is commonly known as the eminentia granularis [37]. This cerebellar architecture holds true across most fish lineages, but in many instances can be further complemented by the addition of cerebelloid structures that enhance species function, behavior, and specialization.

Our early understanding of the cerebellum of fishes has arisen primarily from detailed descriptions of teleost fishes [67]. Teleostei is one of four superorders of the subclass Actinopterygii that also include the superorders Palaeoniscoidei, the Chondrostei, and the Holostei. Collectively, these superorders comprise more than 30,000 fish species, although most modern-day bony fish belong to Teleostei. Thus, in many cases, deviations in the cerebellar plan across fishes are contrasted with the basic teleost morphology and structural design. As mentioned above, the teleost cerebellum shares numerous traits with other vertebrate classes, but at the gross anatomical level, obvious differences are found. Specifically, these include an absence of cerebellar nuclei and well-defined foliations, as seen in the neocerebellum of mammals [28], and the development of a rostral protrusion termed the valvula cerebelli [57], not found in a number of other vertebrates.

The cerebellum of teleost fish and the large majority of fish species is defined by three major structures: the valvula cerebelli, the corpus cerebelli, and the vestibulolateral lobe ([21, 58, 90]; *see* Fig. 2). The valvula cerebelli is the rostral-most structure of the cerebellum, a structure absent in Cyclostomes, Chondrichthii (sharks and rays), and Crossopterygii (ceolacanth; [45]). The valvula projects rostrally as a pouch-like structure [67] into the midbrain ventricle below the superficially located optic tectum. Across species, the valvula cerebelli is variable both in size and shape and in some species can also include a lateral domain [21]. Extreme examples of this variation are illustrated by the extraordinary expansion of the valvula cerebelli in Mormyridae where it has hypertrophied to become a superficial structure that covers the entire surface area of the brain (*further described in section* "Morphological Diversity of the Fish Cerebellum"; [22, 78, 80, 82]). Studies show that the valvula cerebelli receives much of its primary input from the tertiary lateral line system, the tractus mesencephalon-cerebellaris posterior [67]. In Mormyrid fish, this tertiary

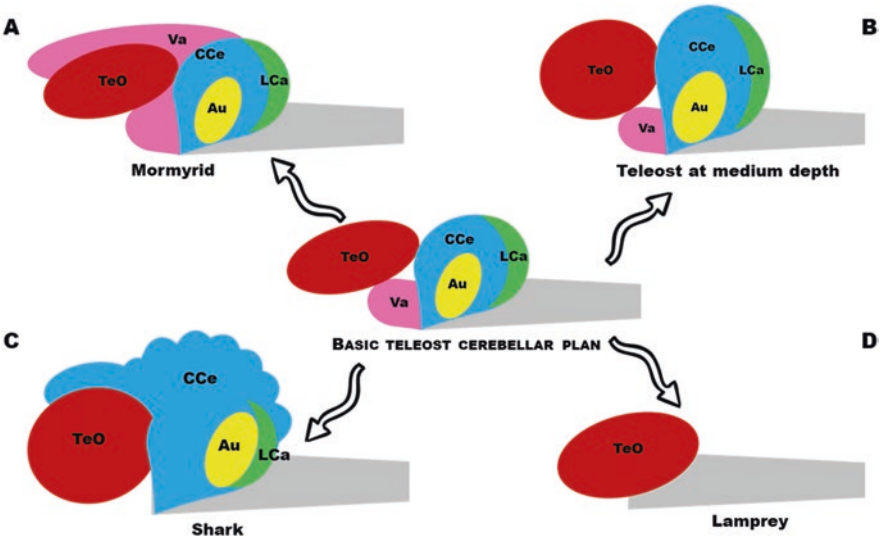

Fig. 2 Schematic representation of differences in cerebellar morphology in reference to the basic teleost cerebellar architectural plan (center). (**a**) Mormyrids display an extreme enlargement of the valvula cerebelli that projects over top of the optic tectum. (**b**) Teleosts specialized to a medium depth in the water column show an enlarged cerebellum and optic tectum. (**c**) Sharks demonstrate an increased degree of foliation of the corpus cerebelli, with this structure projecting over the optic tectum. A valvula cerebelli is absent. (**d**) Lamprey (jawless vertebrate) lacks a true cerebellum. In (**a**–**d**), precerebellar and cerebelloid structures are not shown; gray indicates a simplified view of the medulla oblongata and spinal cord. *TeO* optic tectum, *Va* valvula cerebelli, *CCe* corpus cerebelli, *Au* auricles (eminentia granularis in teleosts), *LCa* caudal lobe of the cerebellum

input is defined by electrosensory projections arising from the lateral toral nucleus of the midbrain [60]. However, evidence for direct lateral line input from the anterior lateral nerve has also been reported [89].

The central portion of the cerebellum consists of the corpus cerebelli, the only portion of the cerebellum visible at the external brain surface of most teleosts [57]. It is considered a tubular structure projecting either rostrally or caudally in different teleost species and is connected to the rhombencephalon by a short stalk, the peduncle [74]. This structure is functionally distinct from the caudal lobe, or vestibulolateral lobe, as its afferent input is not related to the acousticolateral system. In the zebrafish, a species that has been studied in considerable detail with regard to cerebellar development [34, 36, 37], the cerebellar corpus includes only a single folia and reveals the stereotypical anterior extension, the valvula cerebelli [34].

The caudal-most structure of the cerebellum is known as the vestibulolateral lobe [57, 74], considered homologous to the tetrapod vestibulocerebellum. The vestibulolateral lobe of fishes is composed of the eminentia granularis and caudal lobe. For many years, there has been debate as to whether the eminentia granularis in teleosts is equivalent to the auricles of tetrapods [27] or whether the auricles and the granular eminences are truly different structures [75]. Most recently, the former

hypothesis has been supported that this structure is homologous to the auricle of other vertebrates [90]. The vestibulolateral lobe is currently understood to have a strong connection with the central lateral line sensory region.

Histologically, the teleostean cerebellar cortex demonstrates much less organized lamination compared to the more rigid trilaminar cortex of tetropods [57]. The mammalian cerebellum is characterized by three layers that form the cerebellar cortex, including the outer molecular layer with few resident neuronal somas, a monolayer of conspicuous Purkinje cells, and a deep granular layer consisting of a large proportion of small granule cells (reviewed in [18]). This well-defined laminar organization has generally been shown to carry over to nonmammalian vertebrates with the exception of fish and Cyclostomes [44]. These characteristic zones of the cerebellar cortex and their respective cell types are considerably more variable across fish species [57, 74]. To this end, Purkinje cells can be found in the molecular layer, while granule cells can be observed lateral to the Purkinje cell layer. Notably, in teleosts, basket cells are absent [93]; therefore, inhibitory feedback loops are only created by Golgi and stellate cells. In addition, eurydendroid cells appear to further replace deep cerebellar nuclei of other vertebrates [26, 31]. In terms of branching, the dendritic tree of Purkinje cells in fishes is more complex than in amphibians and reptiles, but never as extensive as demonstrated in mammals [74]. In teleosts, the proximal, smooth part of the dendritic tree, which contains the receptive surface for climbing fibers, does not penetrate the molecular layer as seen in mammals. This has been best shown in mormyrid teleosts but is thought to be common across teleost species.

A final trait that sets teleosts apart from many of their land-dwelling relatives is the presence of additional precerebellar and cerebelloid structures. Precerebellar nuclei are neuronal nuclei that extend most of their projections to the cerebellum and therefore are intimately associated with the cerebellum. These structures appear to be unique to fishes. Examination of precerebellar structures in zebrafish shows the existence of two precerebellar nuclei, the nucleus valvula lateralis, and the nucleus paracommissuralis (reviewed in [34]). Specifically, the nucleus valvula lateralis is found in the tegmentum of the midbrain beneath the cerebellar corpus, with its main efferent target being granule cells in the corpus cerebelli and valvulae cerebelli. Conversely, the nucleus paracommissuralis is located in the midbrain and receives input from telencephalon while sending major output to the cerebellum and torus longitudinalis [11, 84].

Unlike precerebellar structures, cerebelloid structures are defined by having a similar architecture and organization as the cerebellum (i.e., cerebellar-like) but are spatially separate [55]. To date, a number of cerebelloid structures have been identified in aquatic vertebrates, largely fishes, including structures such as the medial (i.e., MON; processes lateral line input) and dorsal (i.e., DON; processes input from electroreceptors) octavolateral nucleus, and electrosensory lobes in advanced bony fishes possessing an electrosensory system [4]. However, across all mammals, with the exception of monotremes, the dorsal cochlear nucleus is also considered a cerebelloid structure [4]. Similar to the classic role of the cerebellum in processing sensory–motor input [10], the cerebelloid structures of fishes also process sensory

signals, receive input from the periphery to the deep layers, and parallel fiber input to the molecular layer. Two cerebelloid structures are observed in fishes, including the cerebellar crest and torus longitudinalis. The cerebellar crest is a layer of parallel fibers that cover the lateral line primary sensory brain stem region, originating from a bilateral mass of granule cells caudal to the cerebellar lobe termed the granular eminence [44, 67]. Likewise, the torus longitudinalis is a paired ridge of granule cells located along the medial boundary of the tectum that projects parallel fibers to the surface of the midbrain tectum in the marginal layer [55, 57]. The torus receives input from the valvula cerebelli and is present exclusively in actinopterygian fishes. Both the cerebellar crest cells and torus longitudinalis are defined by unidirectional parallel fibers [57].

Zebrafish provide an excellent example of how in some fish species, the definition of cerebelloid structures can be extended to include a collection of associated structures forming a functional unit or system [34]. In this species, two cerebelloid systems are present. First, in the hindbrain, the medial octavolateral nucleus, along with the eminentia granularis, and the cerebellar crest (crista cerebellaris) are considered to form a cerebelloid system. Here, Purkinje-like cells in the medial octavolateral nucleus extend their apical dendrites to the molecular layer of the cerebellar crest. The cerebellar crest is a molecular fiber layer continuous with the most caudal aspect of the corpus cerebelli. Second, the torus longitudinalis coupled with the midbrain optic tectum forms a second cerebelloid system in zebrafish. Interestingly, in this context, the fiber-rich superficial marginal layer in the optic tectum has been suggested to act as the molecular layer—with the marginal layer receiving parallel fibers from the torus longitudinalis. It is currently hypothesized that the circuit formed between the torus longitudinalis and optic tectum aids in regulating and predicting visuomotor response given that granule cells in the torus longitudinalis respond to visual stimuli as well as to stimuli that evoke eye movements [24, 73]. A more detailed discussion of cerebelloid structures and their proposed function in anamniotes and mammals can be found in work by Bell [4], Bell et al. [5], and Devor [16].

Morphological Diversity of the Fish Cerebellum

Across jawed vertebrates, marked variation in the developed cerebellum exists as a consequence of evolutionary lineages and habitat adaptations. A best example of this is seen by the spectrum of cerebellar size and foliation across amniotes (reptiles, birds, mammals) and anamniotes (fish, amphibians). Only birds and mammals demonstrate the extensive foliation seen at the gross anatomical level, which drastically enlarges the cerebellum of these animals. Rather, fish display considerably developed cerebellums, albeit non-foliated, whereas this structure is much smaller in amphibians and reptiles [57]. However, an incredible feat unique to fish is the impressive level of cerebellar diversity that has been shaped by both evolution and environment. Comparative studies relating fish brain growth with neuroecological

specializations illustrate the impressive "evolutionary plasticity" of brain structures and their ability to become optimized in accordance with the behavioral requirements of the species. In other instances, evolutionary time leading to lineage divergence is likely the mechanism at play that drives cerebellar remodeling. By surveying the morphological variation of the cerebellum across representative fish models and lineages (*see* Fig. 2), new clues regarding how these structural adaptations have come about and their functional role can be explored.

The first true cerebellum of jawed vertebrates is thought to have arisen in cartilaginous fishes [93]. Interestingly, the developmental form of the cerebellum of sharks and rays mirrors that of adult Cyclostomes—characterized by a simple plate-like structure. Notably, only recently has it been confirmed that Agnathans do not possess a traditional cerebellum, but instead cerebelloid structures, including the DON and the MON [93]. As cerebellar development progresses in cartilaginous fishes, a more elaborate cerebellum can be observed. Bilaterally, a rostrolaterally directed lengthening and outpocketing of the caudolateral parts of the cerebellar territory give rise to the paired auricles. At the same time, a dorsally directed evagination of the rostromedial parts of the cerebellar plate forms the corpus cerebelli [67]. The dorsally situated, unpaired, corpus cerebelli ventrally encloses the large ventricular cavity. Meanwhile, the dorsal aspect extends rostrally over the roof of the midbrain and caudally over the lower lip, a band of nervous tissue laterally continuous with the upper leaf of the auricles [67].

In many species of cartilaginous fishes, considerable variation exists in the size and degree of foliation (i.e., wall infolding) of the corpus cerebelli (reviewed in [92]). The presence of varying degrees of cerebellar foliation in chondrichthyans is a feature rarely seen in most populations of teleosts. Early comparative studies of sharks and rays of different sizes have revealed that transverse grooves of different depths can be conspicuously detected on the surface of the corpus as a result of foliation [85]. Smaller species display a very shallow groove, while in larger bodied species deeper grooves and additional sulci subdivide the corpus into multiple lobes. Across vertebrate taxa, increased foliation is thought to accommodate an increase in cerebellar surface area [79]. This in turn allows for an increase in Purkinje cell numbers, thereby enhancing cerebellar processing capacity and facilitating the complexity of cerebellar-dependent functions and behaviors [32, 81, 87]. Histologically, the chondrichthyan cerebellum demonstrates walls with four distinct cell layers: the fiber zone, the granular layer, the layer of Purkinje cells, and finally the molecular layer [67]. Chondrichthyes further feature two cerebelloid structures, including the DON and the MON that join bilateral auricles at the hindbrain [66].

The most primitive bony fishes, namely the Sarcopterygians, further provide an excellent example of how cerebellar diversity closely aligns with major fish radiations. Sarcopterygians are only represented by the sole surviving Crossopterygian, the coelacanth (*Latimeria*), and six extant species of lungfishes worldwide. Studies of *Latimeria* show that its cerebellum can be seen as a well-developed, dome-shaped structure [93], defined by a dorsal evagination of the corpus cerebelli and very large auricles [61, 62]. The impressive size of the auricles is proposed to have developed in conjunction with the highly differentiated lateral line system in this species. The

lateral line system is a sensory system present along the body wall of most fish species composed of thousands of neuromast cells that function to sense hydrodynamic input regarding relative movements between the body and the surrounding aquatic environment [9, 64]. Within the corpus, distinct molecular, Purkinje, and granular cell layers are well organized, a feature uncommon across most modern-day teleosts. Purkinje cells, however, show less laminar organization in the lateral auricles.

Compared to most other groups of fish, the cerebellum of lungfishes is relatively small and has been considered to be more closely related to that of amphibians [93]. The African lungfish (*Protopterus*) is defined by larger paired auricles that overhang the lateral sides of the midbrain, but only a modest sized corpus cerebelli. By contrast, the Australian lungfish *(Neoceratodus*) features smaller auricles but a larger corpus cerebelli, compared to its African relative [29]. However, in both species of dipnoan, a reasonable degree of lamination can be observed representing the three characteristic cerebellar layers: the molecular, the Purkinje, and the granular [67]. Both the coelacanth and lungfish lack an anterior protruding valvula cerebelli, characteristic of teleosts.

Sampling across fish species provides valuable insight regarding the intersection between brain growth, habitat specialization, and structural diversity. Within the wild, studies have shown that the species environment can impose selection pressure on specific regions of the brain, and some excellent examples come from structural modifications of the cerebellum. Some of the best-studied species demonstrating this evolutionary adaptation come from populations of African cichlids [94]. However, in some extreme cases, unprecedented enlargements of the cerebellum have also been observed in distinct groups of fishes as they take on new sensory processing modalities. Most notably, this attribute belongs to electric fishes, such as gymnotiformes and mormyrids, where the cerebellum has largely outgrown the rest of the brain. The role of the environment in shaping structure-specific brain morphology and the behavior processes of fishes has received considerable attention over the years. For example, in both bony and cartilaginous fishes, relative enlargement of the cerebellum has been associated with locomotor behaviors, habitat complexity, swimming speed and agility for prey capture, proprioception, and the acquisition of sensory input [30, 92]. Conversely, the presence of a small cerebellum appears to be a predictor of lower activity levels and a close association with the substrate in fishes [71].

The independent radiations of African cichlids by far provide one of the most accessible, natural experimental datasets as to how habitat stratification impinges upon brain morphology. In all three East African Great Lakes, feeding strategy and microhabitat utilization have been shown to correlate strongly with individual brain structures [30]. Focusing on cerebellar size, it was reported that this structure was considerably enlarged in populations living at medium depths in the water column. This demonstrated that cerebellar morphology was more influenced by microhabitat use rather than feeding type, at least in this population [40]. By contrast, studies of the Antarctic icefish (Notothenoidae; [17]), a perciform relative of cichlids, revealed that the eminentia granularis and crista cerebellaris functionally involved in sensing olfaction and acoustic-lateralis input demonstrated most variability in morphology

across the 32 species examined. These two studies highlight that these related yet isolated populations can display disparate morphological adaptations to their unique environment in order to presumably increase fitness.

This section would not be complete without mention of the extreme cerebellar adaptation in electric-sensing fishes, including Mormyriformes, Gymnotiformes, Siluriformes, and Xenomystinae [93]. These groups are defined by and are unique among ray-finned fishes as having evolved electroreception. Best studied is the gigantocerebellum of the mormyrid. From an evolutionary standpoint, passive electrosensing using ampullary electroreceptors arose first in osteoglossomorph fishes, permitting the detection of external bioelectric fields [14]. Mormyroids became the first species to evolve electric organs and tuberous electroreceptors, allowing for functional electrolocation and communication [80]. Impressively, the cerebellum of mormyrids accounts for nearly 1% to the total body weight of these fishes [59], largely due to the outgrowth of the anterior valvula cerebelli, completely covering the dorsal aspect of the brain [57].

While one might expect extreme differences in the cerebellar neuroanatomical blueprint of mormyrid fishes, with the exception of its conspicuously larger valvula cerebelli compared with other teleosts, it generally follows the same morphological organization [57]. A clear valvula, corpus, and caudal lobe can be observed, but unlike other teleosts, additional subdivisions are present. As reviewed by Meek [57], the valvula includes the valvula strictiori sensu, but also the lobus transitorius and lobe C_1. Of particular note, the corpus cerebelli is distinguished by three additional lobes: C_2 and C_3 directed rostrally, and C_4 directed caudally. Finally, the caudal lobe is differentiated into both the anterior part (connecting to the mechanosensory lateral line lobe) and the posterior part (connecting to the electrosensory lateral line lobe) [6]. Extrinsic connections of mormyrid cerebellum are in line with that of other teleostean cerebella, showing well-defined eurydendroid or giant cells that project to premotor regions [56, 69, 70], along with a highly differentiated precerebellar nucleus lateralis valvulae.

Neurogenesis and Lifelong Cerebellar Development in Fishes

The majority of this chapter has focused on the variation in the adult cerebellar form that accompanies major groups of cartilaginous and bony fishes. In the last 20 years, however, studies of teleost fishes, in particular, have illustrated that cerebellar growth can persist into later life stages as species age. To date, this has not been observed in sharks and rays, although few studies have been performed in these groups. This capacity is made possible as a result of the process of adult neurogenesis, whereby resident neural stem cells in domains of the postnatal brain known as "stem cell niches" continue to generate newborn neurons [95]. Nevertheless, even in mainstream teleost fish models like the zebrafish, it would appear that the degree of brain-wide structural growth and neural stem cell activity shows a sharp decline in older fish [19, 51], suggesting that continuous growth may not be indefinite.

Comparing the relationship between neural stem cell activity and brain growth with aging in the cerebellum of relatively short-lived (e.g., zebrafish ~3 years) and long-lived species such as the sturgeon (~50–80 years) would provide exciting new insight in this field.

Adult neurogenesis is considerably more limited across the mature neuro-axis of the brain in amniotes, but of the small sampling of bony fishes thus far appears to be a highly conserved, widespread trait [23, 46, 97]. In representative teleosts, such as the zebrafish, greater than 16 major domains show constitutively active neural stem cell proliferation that functions to generate de novo neurons [1, 25, 47]. However, upwards of 100 neurogenic sites can be detected [95, 98]. This is in stark contrast to the two main adult neural stem cell niches found in mammals, limited to the subventricular zone of the forebrain and subgranular zone of the hippocampus [63]. In teleosts, although a large number of these stem cell niches border the brain ventricles, exceptions to this rule exist, such as in the cerebellum where throughout life neuro-epithelial-like stem and progenitor cells proliferate at the upper rhombic lip [34]. Importantly, this high neurogenic capacity displayed by zebrafish is mirrored by an equally impressive neuro-regenerative capacity, including the cerebellum [3, 35, 38, 41, 43, 50–52]. Conclusive evidence for ongoing adult cerebellar neurogenesis has been demonstrated not only in the zebrafish, but the goldfish (*Carassius auratus*; [15]), cichlids (*Astatotilapia burtoni*; [53]), killifish (*Nothobranhius furzeri*; [83]), medaka (*Oryzias latipes*; [42]), and electric brown ghost knifefish (*Apteronotus leptorhynchus*; [96]). In mammals, evidence for spontaneous adult neurogenesis in the cerebellum of rabbits has been documented, though this appears to be exclusive to lagamorphs [20].

An outstanding question that remains in the field of teleost adult neurogenesis is why neurogenesis persists beyond embryonic or early developmental stages. In many fish species, structure-specific neurogenesis can be linked to the mode of growth. Most, but not all, teleost fishes are governed by indeterminate growth [65]; thus, the body, including the central nervous system, continues to add more cells as the species enlarges. This has been demonstrated in species of goldfish for many years. In the brown ghost knifefish, governed by indeterminate growth and considered a model of negligible senescence, 75% of all mitotically active cells in the mature brain are located in the cerebellum [96]. In this species, proliferative activity is seen in narrow stripes at the midline of the corpus cerebelli and valvula cerebelli, their neuroanatomical boundaries, and in the eminentia granularis. Similarly, in the zebrafish, the cerebellum proportionally grows more than other major brain structures over the juvenile stage (30–90 days post-fertilization), while the body of the cerebellum housing granule cells demonstrates remarkable growth throughout life [34].

Interestingly, recent studies in the zebrafish have illustrated that this species is characterized by determinate rather than indeterminate growth [8]. This is more reflective of growth limitations seen in amniotes. Nevertheless, this raises the question of why constitutive proliferation is necessary in structures such as the cerebellum throughout life. In-depth studies by Kaslin and colleagues [34, 36, 37] have shown that proliferative activity of stem and progenitor populations of the

cerebellum decline following juvenile development (up to 3 months), but in adulthood those derived from the upper rhombic lip continue to produce granule cells. To this end, following the juvenile stage, no Purkinje or eurydendroid cells are newly generated. This diminished degree of post-embryonic neurogenesis in the cerebellum aligns with a near plateau in zebrafish growth as compared to its close relative, the Giant Danio [8]. It also raises the possibility that these newly generated cells merely aid in maintaining homeostasis by replacing those that undergo cell death. It remains to be seen in models of determinate or indeterminate growth whether these adult stem and progenitor populations of the cerebellum are capable of characteristic responses to environmental input, such as sensory or motor stimuli, that have been shown in other stem cell niches of the adult zebrafish brain [48, 49].

Closing Remarks

The primary goal of this chapter has been to provide a general survey of the diversity of the fish cerebellum and how this structure contrasts many of the features seen in other vertebrate classes. While embryonic development of the fish cerebellum appears highly conserved with its land relatives, the appearance of new cerebellar structures, such as the valvula cerebelli, in addition to precerebellar and cerebelloid structures, highlights important phylogenetic differences across jawed vertebrates. Even across the small sampling of fishes discussed in this chapter, the manner by which the interplay between evolutionary time, lineage divergence, and habitat specialization orchestrate the morphology of the mature fish cerebellum is evident. What is more, in the adult cerebellum of teleosts, the existence of ongoing cell proliferation and neurogenesis raises exciting questions regarding cerebellar function, plasticity, and lifelong structural maintenance.

Moving forward, taking advantage of newer, more tractable laboratory models to study cerebellar ontogeny will unlock yet another level of understanding regarding this structure from a developmental and genetic perspective. For example, the tiny transparent *Danionella translucida*, no more than ~15 mm in adult size, has emerged as an exciting new model in the neurosciences [77]. With many of the same features as the zebrafish, but offering a smaller adult size, the opportunity to perform live *in vivo* imaging of cerebellar growth from fertilization to maturity has arrived. Blending traditional neuroanatomical methods along with new cutting-edge models and molecular tools to study cerebellar development, diversity, and plasticity across fish models offers an exciting future to advance the field of cerebellar neurodevelopment.

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The Role of nNOS/NO on Cerebellar Development in Health and Disease

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Abstract Nitric oxide (NO) is a gaseous molecule that is differentially produced in a variety of mammalian cells with a diverse range of functions. In the cerebellum, neuronal nitric oxide synthase (nNOS) is the predominant enzyme responsible for NO production and boasts the highest expression in this region, more so than in any other region of the brain. This chapter will review the molecular role of nNOS-derived NO in the cerebellum and its contribution to cerebellar development and function, with emphasis on evidence in rodent models. Specifically, attention will be paid to the role of NO in Purkinje neuron development and Bergmann glia function. The connection between cerebellar ataxia and nNOS/NO signaling will also be explored, along with the current literature surrounding NO as a therapeutic for neurological symptoms.

Keywords Nitric Oxide · Cerebellar Ataxia. · Calcium Homeostasis. · Dendrite Morphology. · Excitotoxicity.

Introduction

The cerebellum derives its name from the Latin meaning “little brain” and is often described as a distinct subsection of the brain. Treating this region of the brain as other to the cerebral cortex is understandable, as the cerebellum presents with

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distinct and organized folia that bear little resemblance to the gyri and sulci of the cerebral cortex. Foundational lesion studies from the 1900s determined the overwhelmingly critical role the cerebellum has in motor coordination and fine-tuning motor movements [1]. After the discovery of the intricate interplay between the cerebellum and the cerebrum, the cerebellum has more recently become a target for functions involving spatial recognition and memory [2, 3], as well as emotion and cognitive function [4].

Nitric oxide (NO) is an important and unorthodox neurotransmitter with a wide array of physiological effects on the cerebellum. Once named the "Molecule of the Year" in 1992 for its role as a physiological and pathological cellular signaling molecule, it is undisputed that NO has a critical role to play in maintaining cerebellar homeostasis and Purkinje neuron (PN) function in a variety of ways by regulating synaptic plasticity and glutamate uptake [5–7]. More recently, the link between NO and cerebellar health and disease in rodent models has been made more apparent, as some studies noted the link between mutant mice exhibiting a cerebellar ataxic phenotype and decreases in neuronal nitric oxide synthase (NOS) activity [8, 9]. This is important to consider, as it has been reported that nNOS expression is significantly decreased in the cerebella of aged rats, corresponding to a decrease in learning and memory test performance [10]. Specifically, studies characterizing a neuronal NOS knockout ($nNOS^{-/-}$) mouse model revealed motor impairments and structural changes consistent with some spinocerebellar ataxias (SCAs) seen in humans [7, 11, 12]. This chapter will cover a brief overview of murine cerebellar development; the different isoforms of NOS (with emphasis on nNOS); the synthesis and signaling cascades of NO; the homeostatic functions of NO in the cerebellum during development; and the implications of NO signaling on cerebellar health and disease.

Murine Cerebellar Development: A Brief Overview

Like most structures of the central nervous system (CNS), the murine cerebellum is derived from the anterior portion of the neural tube, specifically the rhombencephalon, typically around embryonic day 11.5 (E11.5) [13]. By E18.5, the first evidence of cerebellar lobules along with PN, granule cells (GCs), and Bergmann glia (BG) expression are apparent [14]. From then, the cerebellum develops into a bilateral structure comprised of two cerebellar hemispheres connected by the vermis, or the midline of the cerebellum.

All GABAergic neurons in the cerebellum—PNs, basket cells (BCs), stellate cells (SCs), Golgi cells, and Lugaro cells—in addition to BG, originate from progenitor cells within the ventricular zone at approximately E10.5–E15.5 [13, 15]. By this time point, PNs, along with other cerebellar GABAergic neurons, migrate toward the pial surface in a radial manner to eventually form the PN monolayer by approximately postnatal day 5 (PD5) [13]. Additionally, the developing cerebellum also contains a second germinal center that originates from the rhombic lip, termed the external granule layer (EGL) [16]. The EGL is located closest to the pial surface

and persists within the cerebellum up until PD14. The rhombic lip gives rise to all glutamatergic neurons of the cerebellum, specifically GCs [13, 17].

Purkinje Neuron Development and Synaptic Innervation

PNs begin their development by establishing axonal connections to cerebellar nuclei and vestibular nuclei during embryogenesis, while an immature form of PN dendritic arborization begins between PD1 and PD3 [18]. At this stage, dendrites appear to be disorganized, extending from all directions, both toward and in parallel to the pial surface. This phase of PN dendritic growth is characterized by abundant climbing fiber (CF) innervation (originating from the inferior olivary nucleus) in a pericellular nest formation, where CF boutons are primarily localized on the PN axon hillock [19]. In this early phase of PN dendritic development, CFs constitute the predominant synaptic innervation to the PN and innervate in a many-to-one fashion [20], while the majority of GCs are located in the EGL in an immature state and with small parallel fiber (PF) extensions [21].

By PD4–PD7, the disorganization of PN dendrites observed in the first 3 postnatal days disappears, and PNs begin to orient their dendritic branches toward the pial surface [22]. In the phase between PD7 and PD28, PN growth constitutes rapid dendritic elongation and synaptogenesis [18, 22]. Importantly, this phase marks the development of the planar orientation of PN dendrites [23]. The rapid PN dendritic elongation also marks a phase for rapid GC migration and maturation into the granular layer (GL) [18]. Likewise, as PN dendrites elongate and form dendritic spines, the once sole glutamatergic innervation provided by CFs is displaced by PFs to form an abundance of PF–PN synapses [24], while redundant CF terminals are eliminated, leaving behind one CF innervating one PN [25].

Bergmann Glia Development

BG, as mentioned previously, are derived from progenitor cells located in the ventricular zone [13]. Undifferentiated BG, simply referred to as radial glia, intimately develop alongside immature PNs and GCs. At approximately E15, radial glia extend long laminar processes toward the pial surface [13]. Then, radial glia somata migrate from the ventricular zone toward the PN layer around the same time frame as early PN monolayer formation occurs, between PD0 and PD7 [26]. In the later phase of PN dendritic development, between PD7 and PD28, unspecialized radial glia differentiate into BG and can be identified by characteristics such as multiple lamellar processes that extend from the soma to the pial surface while also having a close association with PN dendrites [27]. It is during this time of extensive PN dendritic arborization that BG act as a guide to assist in directing PN dendrites toward the surface. As PN dendrites become more elaborate and synapse-dense, BG processes

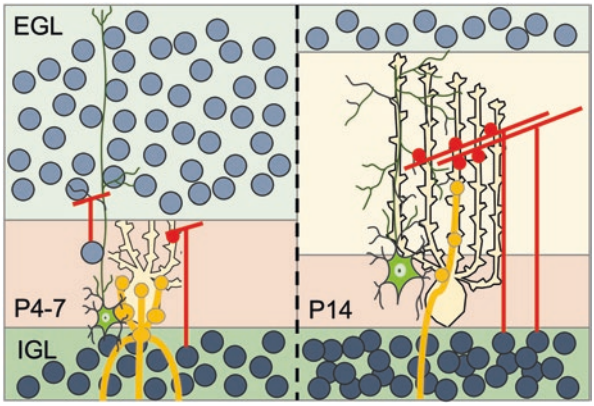

Fig. 1 Early and late stages of PN development. *Left side*: From PD4–7, PNs begin to develop small dendritic branches, and CF innervation dominates the PN, as opposed to PF innervation. The EGL at this point in development is prominent, and BG aid in GC maturation by providing a scaffold to allow for GCs to migrate from the EGL to the GL. *Right side*: In the late stage of PN development (PD7 and onward), the PN quickly develops an elaborate dendritic arbor. By PD14, the EGL is nearly, if not totally, abolished. Elimination of CF synapses occurs, which allows for more PF innervation within the distal PN dendrites. BG processes are more elaborate and closely ensheath glutamatergic synapses upon PNs

will transform their lamellar processes from smooth and linear to fine and elaborate processes that closely ensheath glutamatergic PN synapses [27, 28].

In addition to guiding PN dendritic development, BG processes are critical in assisting GC migration and maturation from the EGL to the GL during the second postnatal week [29, 30]. During this time, BG processes transverse the entirety of the molecular layer (ML), but also a portion of the GL, to ensure appropriate GC migration between the two layers [31]. As GCs migrate through the ML to the GL, they form tight interactions with BG lamellar processes that work to guide GCs to the eventual granule cell layer (GCL) and leave behind developed PFs within the ML [31, 32]. Both the early phase and the late phase of PN and BG development are highlighted in Fig. 1.

Nitric Oxide Synthase Isoforms: Synthesis and Physiological Functions

Nitric oxide (NO) is a gaseous molecule differentially produced in a variety of mammalian cells with a diverse range of functions. There are three enzymes that catalyze the production of NO: neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS), and inducible nitric oxide synthase (iNOS). All three NOS isoforms are classified as oxidoreductases and function as homodimers

physiologically [33]. NO is produced by all NOSs enzymatically by utilizing L-arginine and metabolic oxygen as substrates and producing L-citrulline and molecular water as a by-product [33]. All three isoforms of NOS rely on NADPH as a cofactor and reducing agent, and early studies assessing the localization and function of NOS isoforms relied on NADPH-diaphorase activity [34].

Both nNOS and eNOS are classified as constitutively expressed NOS isoforms. Also, both isoforms produce NO in a calcium-dependent manner [33]. Briefly, intracellular increases of calcium facilitate the binding of calmodulin, which allows for the reductase ability needed to convert L-arginine to NO. Therefore, increases in intracellular calcium levels and/or increases in calmodulin-bound eNOS and nNOS will increase NO production, accordingly [34, 35]. Neuronal NOS—also referred to as brain NOS or NOS1—was the first physiological NOS isoform to be discovered, fittingly within brain tissue. After this discovery, it is now known that nNOS is also localized in the periphery, specifically within skeletal and cardiac muscle, as well as nitrergic nerves that innervate smooth muscle [36]. Furthermore, nNOS is the predominant NOS isoform in the CNS, localized in both neurons and astrocytes of the cerebrum and cerebellum.

NO/nNOS in the Cerebellum

Neuronal NOS is of particular importance in the cerebellum, as this isoform is highly expressed within this region compared to any other region in the CNS [37, 38]. In particular, nNOS is localized to GCs, inhibitory interneurons, and BG, but notably not expressed in PNs [39, 40]. Cerebellar NO has been implicated in many homeostatic functions, including synaptogenesis and plasticity, neurotransmitter release, signal transduction, and cell death regulation [41, 42]. Unlike what is observed in the cerebral cortex, NO acts as an anterograde messenger, often produced in the presynaptic terminals of PFs. Within the cerebellum, nNOS activity and subsequent NO production are mainly triggered by PF stimulation, as nNOS-derived NO is produced in a calcium-dependent manner [43]. After PF stimulation, NO production in the PF is halted by retrograde endocannabinoid signaling through a pathway mediated by cannabinoid-1 receptors on the PF terminal [43, 44].

Another common method of nNOS-derived NO production originates within BG that actively takes up glutamate released from the PF. Glutamate uptake via the sodium-dependent glutamate aspartate transporter (GLAST) on BG triggers a reverse activity of sodium/calcium exchangers, subsequently transporting calcium ions into the BG cytosol [6, 45, 46]. The increased calcium influx in BG, in response to glutamate uptake, triggers the production of NO via the calcium-dependent activity of nNOS.

The nNOS-derived NO can signal endogenously within GCs and BG to play an important role in both migration and maturation during cerebellar development [12, 42]. NO can also function by diffusing into neighboring PNs to influence cerebellar motor learning and memory by modulating synaptic plasticity in the form of

long-term depression (LTD) or long-term potentiation (LTP) [5, 41, 47]. Importantly, nNOS-derived NO within the cerebellum has been previously shown to be critical in promoting PN survival and neuritogenesis during embryonic development in an in vitro environment [42].

Nitric Oxide Signaling Pathways in the Cerebellum

As a highly diffusible molecule, NO production within the cerebellum can produce a localized response with differential effects on a variety of neighboring cells. To carry out a myriad of functions within different cell types, NO is known to act through two common signaling mechanisms: the classical NO–cyclic guanosine monophosphate (cGMP)–protein kinase-G (PKG) signaling cascade or protein modification via *S*-nitrosylation of cysteine residues.

Protein Kinase-G Signaling Cascade

Intercellular NO is commonly known to activate soluble guanylyl cyclase (sGC), a cytosolic enzyme (with observed function on the plasma membrane as well) that is documented to be the only endogenous receptor that uses NO as a ligand. To active sGC, NO binds to a heme group embedded into the enzyme, which results in the hydrolysis of guanosine-5′-triphosphate (GTP) into cGMP [47]. Cyclic GMP acts as a secondary messenger that effectively amplifies the original NO response. Considering the highly diffusible and consequently transient nature of NO, sGC is able to translate the instability of NO into a stable message in the form of cGMP. Additionally, sGC activation results in a 200-fold amplification of the original NO signal, thus preventing the potential cytotoxic effects of NO overproduction [48]. A common downstream effector of cGMP is PKG, a serine/threonine kinase that can phosphorylate a wide variety of proteins within cells [49]. Although PNs themselves do not produce NO, they have been reported to express all of the components associated with the sGC-cGMP-PKG pathway, exclusively activated by NO [49]. Within PNs, critical proteins that are phosphorylated specifically by PKG include α-amino-3-hydroxy-5-methyl-4-isoxazoleproionic acid receptors (AMPARs) and inositol-3-phosphate receptors (IP3Rs), critical for the production of LTD within PNs [40, 50, 51]. The classical NO-cGMP-PKG pathway is illustrated in Fig. 2.

L-Arginine is catabolized by NOSs, including nNOS, through an oxidoreductase reaction to produce NO and L-citrulline as a by-product. As PNs do not express NOS isoforms, NO diffuses from presynaptic terminals like the PF to bind to sGC, initiating the conversion of GTP to cGMP. Cyclic GMP binds and activates PKG, a protein kinase that is able to phosphorylate serine/threonine residues of proteins such as AMPARs, IP3Rs, and cytoskeletal proteins.

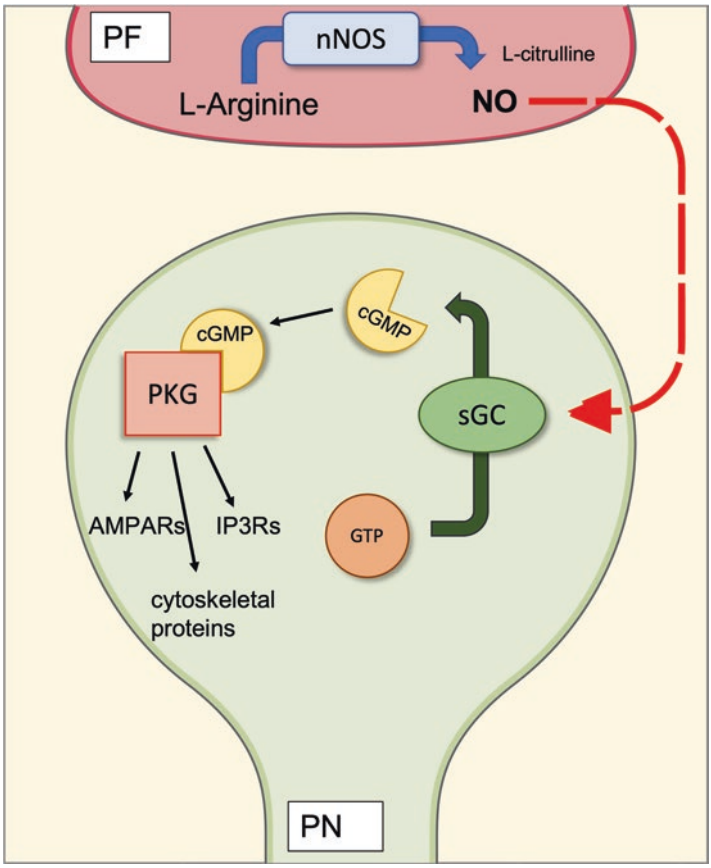

Fig. 2 Activation of PKG via NO signaling cascade

Regulation via S-Nitrosylation

Due to its robust amplification of NO signaling, the sGC-cGMP-PKG cascade is the classical signaling pathway that results in the indirect cellular effects of NO. However, NO can also directly confer reversible post-translation modifications upon cysteine residues of various proteins in the form of *S*-nitrosylation [52]. The transient nature of NO signaling requires the process of *S*-nitrosylation to be relatively quick, and unlike the classical PKG pathway, *S*-nitrosylation requires higher concentrations of NO within the intracellular environment [53]. In order for *S*-nitrosylation of proteins to occur, the cell itself must be in an optimal redox state, specifically within an oxidative environment [53]. Under an oxidative state, cysteine–thiol bonds within proteins transform into thiol radicals that easily react with NO [54]. Within the cerebellum, *S*-nitrosylation is known to affect stargazin, a regulatory protein known to

affect membrane expression levels of AMPARs, cytoskeletal structures like post-synaptic density-95 (PSD-95), and store-operated calcium sensors such as stromal interaction molecule 1 (STIM1) [55–57].

NO and Synaptic Plasticity in the Cerebellum

Synaptic plasticity within the cerebellum is defined as cellular changes that underly the storage of motor memories and the development of important functions, including error-driven motor control and learning, as well as movements associated with the vestibulo-ocular reflex [58, 59]. In general, there exist a variety of ways in which synaptic plasticity may occur, including receptor sensitization, LTP, LTD, and alterations in synapse morphology [60]. The following sections will discuss ways in which PNs display and are affected by synaptic plasticity, with a particular focus on the relationship between NO and PN LTD, as well as associated changes in neuron morphology.

PF–PN Synapse, LTD, and NO

PF–PN synaptic function is crucial in governing the development of fine motor skills as well as coordinated movement, both spatially and temporally [61]. PF–PN synaptic transmission is initiated by the release of glutamate from PF terminals and the binding of glutamate to its receptors located on the PN dendritic spine. In general, glutamate activates ionotropic glutamate receptors, namely AMPARs (PF–PN synapses do not express *N*-methyl-D-aspartate receptors (NMDARs)) to elicit a large and transient depolarization, termed the fast excitatory post-synaptic current (fEPSC) [62, 63]. Also, glutamate activates metabotropic glutamate receptors (mGluRs), specifically mGluR1 expressed on PN dendritic spines, to cause a slow and sustained influx of cations (both sodium and calcium) into the PN, termed the slow EPSC (sEPSC) [62]. The PF–PN synapse is the site for synaptic plasticity in the form of LTP/LTD [64]. The LTD profile functions to weaken PF–PN signaling by modulation of cations entering the PN, specifically calcium, which originates from internal stores from the endoplasmic reticulum (ER) or from the extracellular environment [65]. Unlike in the cerebral cortex, LTD of PF–PN synaptic transmission underlies motor memories, whereas LTP of these synapses is often associated with the extinction of learning some motor skills [5].

Notably, NO plays a large role in facilitating LTD at PF–PN synapses [5, 65]. Experimentally, NO blockade via NOS inhibitors within cerebellar slices abolishes the LTD profile at the PF–PN synapse completely [66, 67]. However, how NO facilitates cerebellar LTD has remained a controversial topic within the scientific literature. The currently favored model describes NO, specifically produced by PF stimulation, diffusing into PN terminals to activate the classical NO-cGMP-PKG

pathway, leading to the hyperphosphorylation of AMPARs on PNs [68]. Therefore, NO production results in decreased AMPAR activity, either through AMPAR desensitization or AMPAR internalization, potentially through either a protein kinase C (PKC)- or a PKG-dependent mechanism [67, 69–71].

Experiments focused on characterizing the profile of sEPSC-related proteins in the cerebellum of nNOS$^{-/-}$ mice revealed a significant downregulation in total mGluR1 protein levels, along with increases in STIM1 protein expression, downstream to the mGluR1 signaling cascade [7]. Following mGluR1 activation, STIM1 proteins oligomerize gate calcium influx through TRPC3 channels [72, 73]. Therefore, changes in expression levels and oligomerization patterns of STIM1 are indicative of calcium dysregulation [74]. Results from the study conducted by Tellios et al. displayed increased STIM1 clustering in nNOS$^{-/-}$ PNs, alluding to aberrant calcium entry through a mechanism of store-operated calcium entry. Indeed, a recent study noted the interaction between STIM1 and NO via *S*-nitrosylation results in the prevention of STIM1 oligomerization and further reduction of SOCE [57]. The absence of NO/*S*-nitrosylation of STIM1 may result in elevated calcium entry through STIM1-gated TRPC3 channels in nNOS$^{-/-}$ PNs, despite decreases in mGluR1 protein expression. Notably, the study conducted by Tellios et al. further discovered significantly elevated levels of a calcium-dependent protease—caplain-1—within nNOS$^{-/-}$ cerebella, further supporting the role of nNOS/NO signaling in cerebellar calcium homeostasis [7].

Synapse Morphology

Accompanying functional synaptic plasticity, structural forms of synaptic plasticity exist in the cerebellum, in which overactivation and under-activation of synaptic terminals can result in changes to synaptic bouton ultrastructure. In general, changes to dendritic spine morphology are often a result of protein synthesis or degradation when building new spines or eliminating unnecessary spines, respectively. The construction of new spines requires increased presynaptic input and activity, and it is agreed upon that intracellular calcium levels play a key role in synapse reorganization [75]. In response to elevated levels of calcium in the postsynaptic terminal, a local change to dendritic spines can occur via calcium-dependent signaling proteins that can alter the dendritic cytoskeleton [76]. Local, fast changes to dendritic spines in response to a stimulus such as glutamate can result in an increase in immature synaptic spines, such as thin or “learning” spines, as opposed to canonical, mature, mushroom-type spines, also known as “memory spines” [77]. Creation of additional thin spines as a result of intracellular calcium levels is often mediated by phosphorylation events through calcium/calmodulin-dependent protein kinases (CaMKs), which are able to alter dendritic structures via modification of cytoskeletal proteins [78]. In the cerebellum, the activity-dependent production of NO is essential for the induction of PF–PN synaptic LTD [66, 79, 80], which contributes to spine morphology [81]. While normal neurite growth relies on optimal levels of

calcium influx during development, chronically increased calcium suppresses neurite elongation and growth cone movement, as discovered in culture [82]. Therefore, a lack of NO signaling can affect intracellular calcium transients within PNs and contribute to dendritic deficits during the early phase of PN dendritic development, as seen in nNOS$^{-/-}$ PNs [7].

In older nNOS$^{-/-}$ cerebella, it was noted that PN dendritic spines appeared to have less mushroom-type dendritic spines and more thin-type spines in relation to wild-type cerebella [7]. Elevated intracellular calcium levels with PNs can result in altered dendrite and spine morphology via calcium-dependent activation of calpain. Notably, further analysis revealed a significant decrease in levels of β-III-spectrin (a substrate of calpain) in the cerebella of adult nNOS$^{-/-}$ mice, along with increased calpain-1 expression when compared to cerebella from age-matched wild type (WT) mice [7]. Given that β-III-spectrin is necessary for the formation of mushroom-like dendritic spines [83], the reduction of β-III-spectrin in the cerebellum of mice lacking nNOS expression may explain the alterations in dendritic structures and synapses in PNs of nNOS$^{-/-}$ mice.

NO, Bergmann Glia, and Cerebellar Development

BG serve as important mediators of both GC differentiation and migration from the EGL to the GL, in addition to PN dendritic and synaptic growth from the PN layer to the pial surface [21, 32, 84, 85]. During early postnatal development (PD0–PD10), BG morphology transitions from distinct smooth lamellar processes that radiate toward the pial surface to rough radial processes that contain outgrowths that work to ensheath PN synaptic connections with PFs and stellate cells [28, 86]. Recent analyses revealed abnormally thick lamellar processes in nNOS$^{-/-}$ cerebella during PD3 and PD7 in comparison to WT [12]. An early study that explored BG morphology in the *weaver* cerebellar mutant mouse reported similarly thick BG lamellar processes, which this group denoted to be the reason behind PN and GC degeneration [87].

As BG lamellar processes mature, radial outgrowths wrap around PN dendritic spines and protect synapses from glutamate-induced excitotoxic damage [28, 86]. Less colocalization between BG processes and PN dendrites in nNOS$^{-/-}$ cerebella might be a driver for the overall decrease in PN mushroom-type spines and overall spine number when compared to WT cerebella [7, 12]. Considering that nNOS is expressed in supporting cells such as BG and GCs, and not PNs [88–90], it is possible that the structural and functional deficits of PNs in the nNOS$^{-/-}$ mice are at least partially the result of aberrant BG growth.

NO and GLAST Regulation

Radial astrocytes, such as BG, abundantly express GLAST as opposed to glutamate transporter-1 (GLT-1) in astrocytes in the cerebral cortex [91]. In particular, GLAST is an important cerebellar glutamate transporter that has a sixfold greater expression level relative to GLT-1, making it the predominant glutamate transporter in the cerebellum [92, 93]. GLAST functions as a co-transporter, in which one glutamate molecule is transported into the BG along with three sodium ions and one hydrogen ion, while one potassium ion is transported to the extracellular space [94]. A significant component dictating GLAST functionality is the frequency and magnitude of calcium transients that occur within the BG in the presence of glutamate. It is well known that PF activity can evoke increases in intracellular calcium within BGs [95–97], and this is postulated to occur by a few mechanisms. First, BG are known to express calcium-permeable AMPARs (AMPARs that lack expression of the GluR2 subunit); therefore, activation of these AMPARs via glutamate induces localized, transient calcium influxes [98, 99]. Calcium transients within BG during glutamate uptake are critical, as studies that have abolished calcium transients mediated by AMPARs have reported BG process retraction as well as decreases in GLAST transcription [45, 100, 101]. A recent study has determined that increases in NO concentrations are proportional to increases in GLAST functionality, measured as relative D-aspartate uptake, in cultured BG [6]. Similarly, glutamate uptake activity within cultured BG has been shown to increase BG expression of nNOS, suggesting an intricate interplay between glutamate concentrations, nNOS expression, and GLAST activity [90]. More recently, a study by Tellios et al. showed that cultured BGs isolated from $nNOS^{-/-}$ cerebella exhibit decreased trafficking of GLAST to the plasma membrane compared to WT BG, resulting in less GLAST activity, measured by live cell imaging of intracellular calcium and sodium [12].

NO may also indirectly affect GLAST function by modulation of the calcium transients within BG during glutamate uptake. Specifically, GLAST activity is closely coupled to the sodium/calcium exchanger, resulting in an overall increase in intracellular calcium. Interestingly, multiple studies have demonstrated the ability of NO to cause a reversal activity of the sodium-calcium exchanger, resulting in increased calcium influx into the BG [102–104].

Cerebellar Disorders and Implications

Cerebellar dysfunctions have increasingly begun to be included in a variety of neurological disorders, including autism, Alzheimer's disease, and Parkinson's disease [105–107]. The following section will describe canonical clinical cerebellar disorders and their association with PN function, PF–PN synaptic transmission, cerebellar calcium dynamics, and NO signaling.

Spinocerebellar Ataxia

Spinocerebellar ataxias (SCA) are a group of rare hereditary ataxias that lead to degenerative changes within the cerebellum and in some cases, the spinal cord [108]. Over 40 SCAs have been identified thus far, each with their characteristic genetic mutation, with a global prevalence rate of 0.3–3 per 100,000 per capita [108, 109]. Symptoms of the disease along with the perceived severity can vary depending on the type of SCA as well as the age of onset and mainly include uncoordinated gait, impaired hand, and eye movements, as well as poor speech formation and cognitive deficits [109–111]. At the physiological level, the cerebellum often shows increased atrophy as well as degeneration and loss of PNs [112, 113]. In human SCAs, mutations in *ATXN*, *SPTBN2*, *CACNA1A*, *ITPR1*, and *TRPC3*—genes encoding ataxin-1, β-III-spectrin, CaV2.1, IP3 receptor, and TRPC3—are affected in SCA1, 5, 6, 15, and 41, respectively, and are crucial in maintaining PN viability and functionality [62, 114–118]. Specifically, these gene mutations are similar in that they alter calcium ion dynamics within the PN, consequently resulting in PN degeneration and loss [119–121]. Results discovered in the $nNOS^{-/-}$ mouse model also harbor similar features to those discovered in human SCAs, including decreased protein expression of β-III-spectrin and impairments to the mGluR1 pathway [7]. It is understood that calpain activity can induce the degradation of neuronal cytoskeletal proteins, including α- and β-spectrins, as well as IP3 receptors, which are implicated in the progression of multiple SCAs [122–128]. Therefore, the knowledge that a lack of nNOS-derived NO signaling may mimic a similar phenotype to that of human SCAs could provide foundational knowledge in better understanding the progression of SCA progression as well as other movement disorders associated with the cerebellum.

Episodic Ataxia

Episodic ataxias (EAs) are similar to SCAs in that they are both neurological conditions originating in the cerebellum that affect movement coordination [129]. EAs are also relatively rare disorders, affecting less than 1 in 100,000 individuals [129]. Unlike SCAs, which present with chronic symptoms, EAs are characterized by transient, periodic bouts of ataxia, along with secondary symptoms including seizures and slurred speech [130]. Like SCAs, there are multiple types of EAs, presenting with various genetic mutations, in particular *SLC1A3*—a gene encoding for GLAST in humans and leading to the progression of EA6 [131, 132]. The progression of both SCA and EA is affected by changes to BG and GLAST expression and functionality. Although SCA1 is caused by a mutation in the *ATXN1* gene, reports have shown that the loss of the protein ataxin-1 can negatively affect the morphology of BG, while analyses of humans with SCA1 revealed overall less BG compared to healthy controls, which suggests that stimulating BG activity and proliferation might be beneficial for treating this form of SCA [117, 133]. Although the severity

of the PN deficit is currently unclear in clinical cases of EA6, characterization of the GLAST$^{-/-}$ mouse model showed a large detriment to PN synaptic transmission and development [30]. Understanding the relationship between NO and GLAST functionality may bring to light novel ways to increase BG activity and GLAST expression to ameliorate motor deficits seen in various ataxias.

NO as a Therapeutic: Good or Bad?

NO is a hotly disputed molecule in terms of its role in physiological and pathological contexts. In general, chronically elevated NO concentrations have proven detrimental in a variety of neurological pathologies, such as stroke, multiple sclerosis, and glaucoma [134–137]. In pathologies such as stroke, NO levels increase as a result of excitotoxicity and subsequently result in decreased neuron survival. Specifically, NO as a free radical can oxidize and form reactive oxygen and nitrogen species in high concentrations that are detrimental to cell metabolism and eventually induce apoptosis [138]. In excitotoxic environments within the cerebral cortex, NMDARs are coupled with the production of NO via nNOS, in that overactivation of NMDARs on neurons results in a chronic influx of calcium, consequently causing the overactivation of nNOS and the overproduction of NO [139, 140]. Additionally, stressed or dying cells can release cytokines such as TNF-α, which can trigger the expression and activation of iNOS within neurons and supporting cells [141]. NO production via iNOS activity occurs as a burst effect, releasing higher levels of NO in a shorter period of time, and has often been deemed the culprit of pathologically elevated NO levels [142].

Despite the detrimental effect NO may have at pathologically high concentrations, physiological levels of NO are crucial in maintaining normal neuronal structure and function. Importantly, a lack of NO can produce effects that are equally as harmful to neuronal homeostasis as an excess of NO, as demonstrated by recent articles characterizing cerebellar development in nNOS knockout (nNOS$^{-/-}$) mice [7, 12], as well as in neuropathologies such as Huntington's disease and to some degree schizophrenia and depression [143, 144]. In the cerebellum specifically, PNs have the added benefit of not expressing NMDARs on PF–PN synapses, which may alleviate the detrimental effects of excess NO production in these cells. However, maintaining physiologically appropriate levels of NO via exogenous application may be difficult, as NO is a highly diffusible and transient molecule. Although there have been recent advances in optimizing the slow release of NO for neurological targets, using NO as a widescale therapeutic may be tricky [145]. As mentioned previously, multiple animal models of SCA have identified a decrease in cerebellar NOS activity, highlighting a common deficit associated with this pathology [8, 9]. In order to avoid the unpredictability of exogenous NO supplementation, clinical therapeutics often target downstream effectors of NO, such as cGMP or PKG, in order to elicit similar effects. Gaining a better understanding of what interacts with NO in the cerebellum is the first step in conceptualizing therapeutics to combat motor deficits.

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Developmental Disorders of the Cerebellum and Neurotrophic Factors

Leila Pirmoradi and Shahla Shojaei

Abstract The cerebellum plays a central role in motor control and 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. The role of neurotrophic factors on the growth, proliferation, differentiation, and arborization of neurons and thus neurodevelopmental disorders has been established and investigated for decades. Numerous studies have shown changes in the level of a neurotrophic factor in the serum or tissue and alterations in their receptors and components of their signaling pathways in these neurodevelopmental diseases. This chapter provides a brief overview of neurotrophic factors and their role in cerebellar development. We also focus on the functions of the neurotrophin system in developmental disorders and diseases of the cerebellum.

Keywords Attention deficit hyperactivity disorder · Autism · Developmental dyslexia · Joubert syndrome · Ataxia · Rett syndrome · Joubert syndrome · Dandy–Walker malformation · Brain-derived neurotrophic factor · Nerve growth factor · Neurotrophins · Transforming growth factor-beta · Neurotrophic cytokines

Introduction

The cerebellum coordinates motor function and preserves equilibrium [63, 158]. It is also an essential region of the brain for behavior and cognition in all aspects, including language, memory, sleep, attention, and spatial and social-emotional

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processing [3, 45, 141, 158] [reviewed in [110, 157]. Early damage to the cerebellum results in more drastic and long-lasting effects on movement and cognition [168]. Early abnormalities in cerebellar function and regulation result in developmental disorders such as autism, ADHD, developmental dyslexia, and Joubert syndrome [79, 106, 168]. Many studies have investigated the molecular mechanism of cerebellar development, and the role of neurotrophic factors is well known [85, 188]. In the cerebellum, neurotrophic factors have a crucial effect on the generation, differentiation, and proliferation of different neuronal cells such as granule cells, Purkinje cells, and glia [85, 188]. Dysregulation of their pathways was associated with developmental disorders in the cerebellum [39, 146, 148, 149].

Neurotrophic Factors

Neurons and glial cells are dependent on growth factors for their normal function, differentiation, and survival [44]. The neurotrophin family of peptides was the first discovered family of growth factors that affect the central nervous system (CNS) [103]. Neurotrophic factors modulate the formation of the CNS by affecting the development and differentiation of neuronal cells in utero [103]. These proteins are also expressed throughout life and have central roles in regulating the function and survival of neurons and glial cells [96, 97]. Receptors for these factors have also been discovered in many tissues. They mediate a wide range of actions, including the morphogenesis of kidneys and differentiation of vessels and immune cells [48, 152, 159]. Neurotrophic factors have been classified into three groups: neurotrophins (NTs), the transforming growth factor-beta (TGF-β) superfamily, and neurotrophic cytokines (Fig. 1) [82].

Neurotrophins

Neurotrophins (NT) are the best-studied neurotrophic factors, and their concentration changes play a central 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, memory, and regeneration processes by facilitating synaptic transmission and plasticity. The NT family was first introduced by discovering nerve growth factor (NGF). Brain-derived 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) [82]. 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

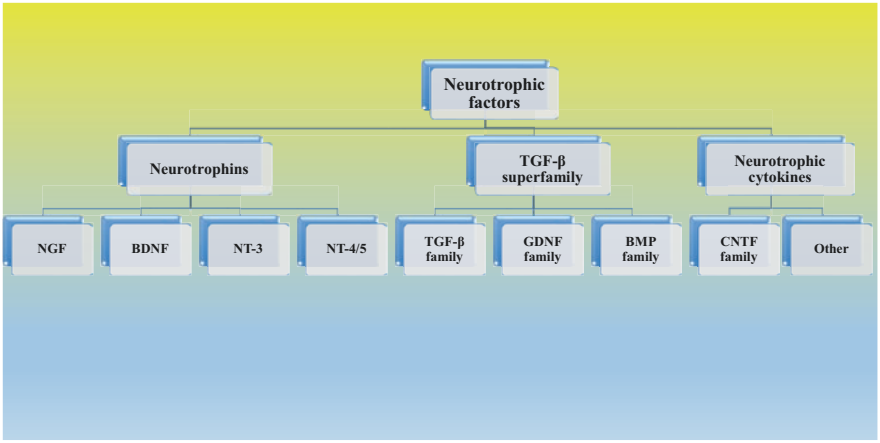

Fig. 1 Classification of neurotrophic factors. Neurotrophic factors are classified into three main groups: (1) neurotrophins, (2) transforming growth factor-beta (TGF-β) superfamily, and (3) neurotrophic cytokines. Each of these groups is divided into their subgroups. Nerve growth factor (NGF), brain-derived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

NT preferentially binds to its respective Trk receptor, resulting in Trk dimerization and subsequent tyrosine autophosphorylation, activating intracellular signaling pathways. NGF binds to TrkA, while BDNF and NT4 bind to TrkB, and NT-3 binds to TrkC. Although pro-NTs cannot activate Trk receptors, they activate p75NTR to promote cell apoptosis via Rac1/c-Jun N-terminal kinases (JNK) pathways (Fig. 2) [82, 132]. Additionally, p75NTR can form a heterodimer with Trk receptors, lowering their affinity and promoting survival through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. Disruption of the p75NTR signaling pathway has been observed in several autoimmune diseases [82].

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 and 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 essential role in CNS function can be modulated by Trk signaling through activation of small Rho GTPases. Trk signaling also improves mRNA translation globally by inducing the phosphoinositide 3-kinase (PI3K)–AKT pathway and transcription of activity-regulated genes such as FOS and ARC [132]. BDNF helps myelination of the CNS in physiologic conditions and even improves myelin injury [reviewed in [50]]. The role of BDNF/TrkB signaling in the learning of fear and also other signaling pathways, including proBDNF/p75NTR, NGF/TrkA, and NT-3/TrkC in the amygdala, has been proposed (reviewed in [116]).

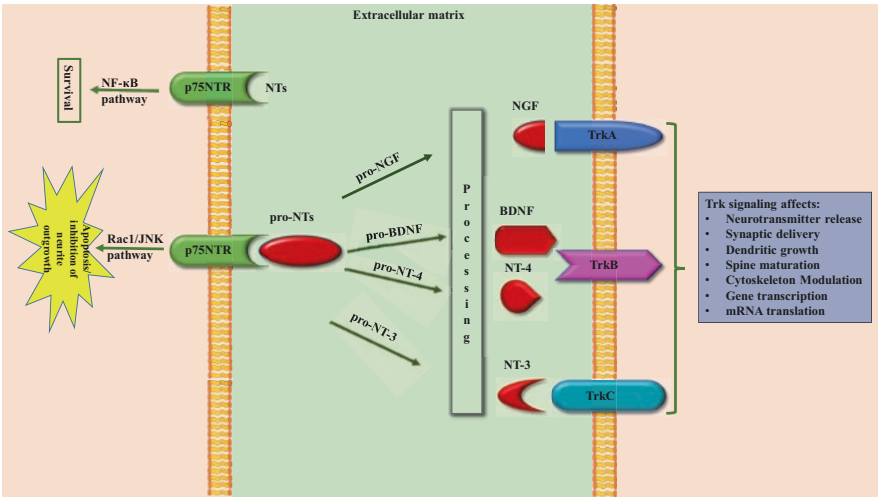

Fig. 2 Pro-neurotrophins (pro-NTs) activate p75NTR to promote cell apoptosis via Rac1/c-Jun N-terminal kinase (JNK) pathways. Upon processing to their cognate mature NTs, they interact to their specific Trks. NGF binds to TrkA, BDNF and NT4 bind to TrkB, and NT-3 binds to TrkC. NTS can also interact with p75NTR with lower affinity and promote survival through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. TRK signaling exerts a diverse effect on the nervous system

Some of the climbing fibers are eliminated during development in the cerebellum, and only one of them is strengthened, and this pattern is crucial for the formation of a practical neural circuit [66]. BDNF released from Purkinje cells helps this synapse deletion in climbing fibers via TrkB retrogradely [30]. BDNF-TrkB, as a crucial signaling pathway, is damaged in peroxisome biogenesis disorders leading to an abnormality during cerebellum development [2].

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 various functions such as controlling the cell cycle, effects on differentiation, early development regulation, extracellular matrix formation, hematogenesis modulation, and immune reactions. The TGF-β superfamily comprises approximately 30 proteins in mammals that are divided into three families: TGF-β family, glial cell-derived neurotrophic factor (GDNF) family, and bone morphogenic protein (BMP) family that each is subdivided into its members (Fig. 1) [64, 82]. 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 ([180], Subramaniam, Strelau et al. 2008, [144]). 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 affects peripheral neuron morphogenesis [21]. GDNF assists with Purkinje cell function and survival in the cerebellum [20, 178]. Purkinje cells are the target of the molecular layer interneurons, which express GDNF during development via GDNF receptors GFRα1 and RET. Molecular layer interneurons are necessary for cerebellar-dependent motor learning [160]. On the cerebellar granule neurons, GDNF has a protective effect against TGF-β cytotoxicity [170].

The insulin-like growth factor (IGF) as a mitogenic protein consists of IGF-I, IGF-II, and six binding proteins (IGFBP-I–IGFBP-6). Insulin-like growth factor-binding protein-2 (IGFBP-2) is a polypeptide that acts as a neurotrophic factor, expressed in the cerebellum and other brain parts. IGFBP-2 has a role in brain development and neuronal plasticity, leading to cognitive functions like spatial learning and memory [reviewed in [87]].

Neurotrophic Cytokines

Neurotrophic cytokines are a group of neurotrophic factors divided into 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 the survival and differentiation of sensory, sympathetic, and motor neurons, thereby influencing the development and maintenance of the nervous system [134] (Fig. 3).

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 [139]. 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 [156]. NGF receptor expression in Purkinje cells shows the importance of NGF in cerebellar development [33, 89]. Increased granule cell precursor proliferation and migration are characteristic features of postnatal cerebellar cortex development [83]. 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 [177]. Other studies discussed the survival effect of NT-3, NT-4, and BDNF [85]. 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 usually 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, which likely is the mechanism

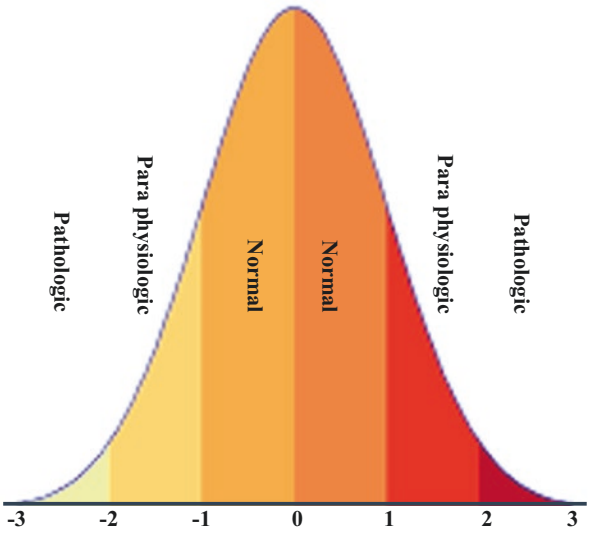

Fig. 3 Correlation of the level of neurotrophins with psychiatric disorders. The level of neurotrophins in the blood and neural tissues has been proposed to determine vulnerability to psychiatric disorders

involved in neurodegenerative diseases [52]. CNTF has a similar effect on Purkinje cell survival [85]. There was some controversy about the survival effect of BDNF in these cells and, more specifically, the impact of this factor on their dendritic development [85]. Kapfhammer and colleagues reported 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.

During cerebellar development, p75NTR is also expressed in the proliferating granule cell precursors (GCPs). Lack of p75NTR can lead to the GCP cell cycle speed-up, the GCP proliferation delay, and excess glutamatergic input to Purkinje cells that is resulted in abnormal behaviors [195].

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 [93]. Instead, Kapfhammer et al. showed the survival-promoting effect of BDNF in these types of cerebellar cells [85]. Environmental enrichment (EE) partially affects the cerebellum via the 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 in 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 [7]. EE improves motor function after cerebellar damage in rats, attributed to regeneration processes caused by NTs [59]. 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 in rats in the ground control group [151]. Conversely, in neonatal rats exposed to hypergravity, there was a significant decrease of NGF expression in the neonates' cerebellum during birth on a postnatal day. However, the basic mechanisms by which NT acts in this condition are not yet known [148, 149].

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 is the most important in cerebellar development. Mutation and dysregulation of this pathway are accompanied by medulloblastoma, a CNS tumor originating from the cerebellum [9, 145].

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 a part of the brain that has a central role in emotion, cognition, behavior, and social interactions [16, 45]. Thus, any damage to the cerebellum early in development could profoundly impact movement, cognition, and learning. Autism, ADHD, and developmental dyslexia are well-known developmental disorders of the cerebellum [168].

Attention Deficit Hyperactivity Disorder

Dysfunction of the cerebellum is a characteristic of some developmental disorders such as ADHD [168]. Studies implicate frontostriatal and frontocerebellar catecholaminergic circuit disorders in ADH pathophysiology. 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 [56]. Many studies on the pathogenesis of ADHD have focused on and confirmed the genetic association of the BDNF gene [92] or its polymorphisms [11, 19, 29, 90] with ADHD. A recent large-scale DNA sequencing study supported this association [72]. 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 [133]. However, a meta-analysis conducted on four European people refuted the involvement of BDNF Val66Met polymorphism with the pathogenesis of ADHD [150]. Recently, another study was performed to address this controversy [196].

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 [164]. A later study by the same group confirmed these findings [165], while Scassellati et al. showed no difference in the serum BDNF level between healthy and affected groups using the same samples [155]. A study enrolling Caucasian adult ADHD patients showed that these patients had decreased serum NT levels compared with the control group [35].

The role of NGF and its receptor (NGFR) has been shown in patients with ADHD [12]. NGF exerts a trophic and functional role in the basal forebrain cholinergic neurons, which are involved in attention [56, 154, 171]. Serum NGF levels were higher in drug-naive ADHD patients at childhood [67]. Bilgic and colleagues showed that serum NGF and BDNF levels in Turkish children were not significantly associated with ADHD, while serum GDNF and NT-3 were higher in the patient group, although it is suggested that the NT-3 level was not associated with the severity of ADHD [21]. Higher plasma GDNF in this disorder is related to being impulsive; a lack of attention and hyperactivity has been recognized. In addition, FGFR has a role in ADHD etiology that acts probably via activation of FGFR1b and FGFR2b [reviewed in [56].

Autism Spectrum Disorders

Autism spectrum disorders (ASDs) are neurodevelopmental disorders that impair communication and social ability [reviewed in [182]]. Both genetic [84, 86, 173, 187] [reviewed in [32, 37] and environmental [27, 47] [reviewed in [51]] factors are involved in etiology of ASD, and cerebellar involvement in ASD has been recognized [reviewed in [62, 70, 179]] (see chapter "Neurodevelopmental Disorders of the Cerebellum: Autism Spectrum Disorder"). In addition, it is suggested that sex responds differently to environmental factors, including immune response to infection. It has been shown that the BDNF expression level was lower in the cerebellum of postnatal male rats, and the IL-6 expression level was higher in the female after *E. coli* infection [129].

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. It induces other aspects of cognitive deficits [163]. Measurement of neurotrophin mRNA levels such as NGF, BDNF, and NT-3 and their respective TRK receptors in newborn rats infected with the Borna disease virus showed no alterations in the cerebellum. However, there were increased apoptotic cells in the cerebellar granular layer and loss of Purkinje cells [198]. 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 [124].

Similarly, in a postmortem study, the cerebellar NT-3 level was higher in ASD patients than normal controls [148, 149]. Another neurodevelopmental rodent model that mimics prenatal immune activation as an environmental risk factor for ASD and schizophrenia is the maternal lipopolysaccharide (LPS) exposure rat model [192]. LPS-treated pups on P21 show increased levels of cerebellar NT-3 [163].

Vitamin D is another factor that adjusts the production of neurotransmitters and growth factors, including NT-3, NGF, and GDNF. Vitamin D receptor exists in the CNS, and its deficiency has been reported in ASD (reviewed in [107]).

Levels of neurotrophins are increased in the blood of children with ASD [104]. The elevated levels of serum NGF and other neurotrophins can be associated with the development of ASD and mental retardation later in childhood [14]. A variant type of BDNF has been found in autistic families in addition to increased blood levels of this neurotrophin in ASD children. Thus, BDNF has been proposed as a therapeutic target for the treatment of ASD due to its critical involvement in the development of ASD (reviewed in [69]) [13, 54, 88]. Conversely, another review proposed a decreased blood level of BDNF as a marker for ASD prediction and prognosis (reviewed in [40]). Sadakata and colleagues reported that transgenic knock-out mice with missing Ca^{2+}-dependent activator protein for secretion 2 (CAPS2), a protein involved in NT release, were susceptible to autistic features [146, 147]. Nickl-Jockschat et al. discussed that altered neurotrophin levels are a pathological mechanism. As mentioned earlier, pro-NT has more affinity to activate p75NTR and subsequently more apoptotic cell death. Therefore, the changes in the ratio of pro-NT to NT can result in some pathological aspects [125].

Immunological stimuli that cause BDNF release from microglia are crucial for cell survival and neuronal differentiation. Intracellular Ca^{2+} signaling is vital for microglial functions in neurodevelopmental disorders such as ASDs [reviewed in [120]]. Neurotrophins such as NGF and BDNF play a role in dendritic morphology [61]. Dendritic shape abnormalities and more enormous dendritic spines have been detected in ASD patients. The cerebellum and inferior olive size variations have been reported in postmortem examinations of the brains of ASD patients. These anomalies in dendritic branching happened in other neurodevelopmental disorders linked to ASD, such as Fragile X and Rett syndrome (RTT) [34]. RTT is a genetic disorder considered to be an ASD previously [18] (see chapter "Epigenetics and Cerebellar Neurodevelopmental Disorders"). For years, there was a debate on RTT classification as an autistic developmental disorder. In 2013, the American Society of Psychiatry changed the classification of RTT and removed it from the ASDs because of its unique molecular basis [1].

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 [26, 136]. MeCP2 alters the expression of many genes in the cerebellum [17]. While serum BDNF levels in RTT girls and a normal group are similar, BDNF protein levels are reduced in RTT brains [18]. Reduced or unaltered NGF in the cerebellum and other brain regions has been reported [156]. Calamandrei and colleagues displayed that serum NGF levels decreased with age [24].

Insulin-like growth factor-1 (IGF-1) is another factor that is changed in ASD, and its role is the alteration of IL-6 expression and microglial function (reviewed in [143]).

Ataxia

Cerebellar ataxias are neurological disorders that can affect the vermis, paravermis, and hemisphere of the cerebellum during development [111] (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 [91]. Neuronal loss in the cerebellar nuclei and Purkinje cell layer has been reported in MJD [91]. Since p75NTR has a vital 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 [140]. Although extrinsic BDNF can protect Purkinje neurons and delay motor deficits onset in spinocerebellar ataxia type 1 (SCA1) model mice [117], it did not change gene expression in Purkinje cells [162].

Because NGF and its receptor TkrA exist in human cerebellar neurons and are involved in the development and stability of the cerebellar connections, NGF therapy may improve symptoms in patients with SCA3 [174]. Jones and colleagues reported that mesenchymal stem cells enhance the survival of Purkinje cells by expression of BDNF, NT-3, or GDNF [81]. 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. In contrast, BDNF mRNA in the cerebellar granule cell layer was reduced [138]. In SCA6, decreased BDNF mRNA expression and altered BDNF protein levels in Purkinje cell dendrites have been shown [172]. Administration of TGF-β1 inhibits TNF-α release and prevents microglial activation leading to neuroprotection in rats with induced cerebellar ataxia [25]. In addition, it has been reported that cerebellar ataxia is likely resulted from Vitamin B deficiency and gut microbiota have an important role in vitamin B synthesis [reviewed in [105]].

Ethanol Neurotoxicity

Ethanol toxicity is when cerebellar cells are vulnerable to ethanol's neurotoxic effects during development (see chapter "Teratogenic Influences on Cerebellar Development"). In addition to the 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 [71, 183] and white matter degeneration in the cerebellum [183]. Duc and colleagues reported that prenatal granular neurons exposed to ethanol in vitro are more sensitive to hypoxic/hypoglycemic conditions. 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, affecting granule cell migration and altering Purkinje cell function (Reviewed in [65]). The least amount of embryonic alcohol exposure decreases BDNF- and NCAM (neuronal cell

adhesion molecule)-positive cells in all brain areas, but synaptophysin expression that indicates synaptic connections does not change in the cerebellum and preoptic area [108].

BDNF, NT-4, and TrkB are highly expressed in the cerebellum. There is evidence that developmental outcomes of ethanol exposure are mediated by the alteration of neurotrophins [74] in an area- and time-specific manner (reviewed in [41]). Furthermore, BDNF variations were associated with a greater volume of cerebellum and gray matter in alcohol-dependent families [77]. Prenatal ethanol exposure caused a decrease in BDNF expression in the embryonic rat brain [166]. Ethanol reduces BDNF and NT-3 secretion in the neonatal rat cerebellar granule cells. Reduced neurotrophin levels increase after treatment by vitamin E [75]. Ethanol influences neurotrophin receptor expression, including TrkA, TrkB, TrkC, and p75NTR. After ethanol treatment in the early postnatal rat cerebellum, a reduction of these receptors was reported [123].

Ethanol prevents BDNF activity in the cerebellum, leading to damage to Purkinje cells, which may occur via impairment in the regulation of BDNF, TrkB receptor, or related signaling pathways [58]. BDNF is also necessary to develop and migrate cerebellar granule cells in the postmitotic period [22]. In vitro experiments on cerebellar granule cells showed that ethanol inhibits the BDNF-stimulated phosphorylation of extracellular signal-regulated protein kinase (pERK) activation in neurons [127]. 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 AP-1 activity linked to BDNF [98]. This change in BDNF signal transduction reflects developmental abnormalities from ethanol consumption [127]. Reduction in BDNF is associated with sensitivity to neural cell degeneration [38]. It has been shown that the levels of BDNF, TrkB receptor mRNA expression [100], TrkC receptor [101, 102], and NGF in the cerebellum decrease on postnatal day 4 or 5 [73].

NGF plays a critical role in developing different brain parts, such as the cerebellum. Studies have shown that ethanol exposure in neonatal rats reduces current NGF receptor levels in Purkinje cells, like other neurotrophic factors [43]. Purkinje cells showed the most harmful effects of ethanol during early neonatal development of the cerebellum [101, 102]. This effect influences neurotrophin signaling [73]. It upregulates pro-apoptotic molecules [74, 122], which cause Purkinje cell loss via apoptosis [101, 102]. 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 cerebellar granule neuron outgrowth. However, the effect of ethanol on BDNF-dependent axon growth and the ERK1/2 pathway in cerebellar granule cells is controversial [28]. Studies in a mice model with fetal alcohol spectrum disorder (FASD) showed that the anterior lobe of the vermis is widely affected, and lobules I–V, which are responsible for sensorimotor functions and crus II and lobule VIIB that control cognitive functions, are damaged [reviewed in [119]]. Higher TrkA receptors and p75NTR deliver more stability against degeneration in the posterior part of the cerebellum [153]. 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 [60]. 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 [49].

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 [114]. These researchers also observed a preventive effect of exogenous GDNF against apoptotic cell death signaling caused by ethanol treatment in a cellular model [115]. Then, Chen and colleagues showed that GDNF, netrin-1, and L1, an adhesion molecule in neural cells, have converging effects on activating the SFK-cas-ERK1/2 pathway to promote axonal outgrowth. Ethanol disrupts this pathway and inhibits axonal arborization in cerebellar granule cells [28].

Medulloblastoma

Medulloblastoma is the most common pediatric brain tumor in the cerebellum of infants and children [145, 185] (see chapters "Primary pediatric brain tumors of the posterior fossa: Part I" and "Primary pediatric brain tumors of the posterior fossa: Part II A comprehensive overview of medulloblastoma"). Marchetti et al. suggested a critical role for NTs and their receptors in the invasive feature of human medulloblastoma [109]. Although, as previously mentioned, Trks are essential factors for neuronal survival, NGF/TrkA signal transduction is accompanied by suppression of medulloblastoma cell proliferation [8] and induction of cell death [31, 55, 128]. Interaction of the cytoplasmic adaptor protein CCM2 with the TrkA receptor is necessary for this pathway. The mediator of TrkA-CCM2 death signaling in medulloblastoma cells is STK25, a germinal center kinase class III (GCKIII) kinase (STK24, STK25). Reduction of STK25 prevents medulloblastoma cell death induced by NGF-TrkA [36]. Another study involving a cellular model of medulloblastoma reported induction of cell death after activation of TrkA by NGF through macropinocytosis [99]. Valderrama et al.'s findings confirmed that induction of TrkA expression resulted in either medulloblastoma cell differentiation or apoptosis [181]. Whole-genome microarray analysis revealed that TGFβ is a potent factor influencing tumor cells' progression and metastasis in medulloblastoma [9]. Gate and colleagues showed that obstruction of TGFβ signaling almost eliminates T regulatory cells and improves CD8(+)/killer cell function to eradicate tumor cells [57].

Thomaz showed that the TrkA and TrkC activation lead to cell death, while TrkB activation leads to medulloblastoma growth. Then, the factor that inhibits TrkB can act as an antitumor [reviewed in [176]]. ANA-12, as a selective TrkB inhibitor, demolishes human UW228 and D283 medulloblastoma cells proliferation by

decreasing extracellular-regulated kinase (ERK) activity, accelerating apoptosis and increasing signal transducer and activator of transcription 3 (STAT3) expression [175].

Schizophrenia

Schizophrenia, which is classified as a late-onset neurodevelopmental disorder [34], is a hereditary (80%) chronic mental disease [80] with cognitive abnormalities [126]. Brain imaging studies have suggested the involvement of cortico-cerebellar connections in cognition [42]. Researchers have suggested that schizophrenia may be related to cerebellar anomalies [46], including a size and density decrease of Purkinje cells and remodeling of synaptic protein expression in the cerebellum [137]. There is growing evidence of a role for neurotrophin in the pathophysiology of schizophrenia [5, 167]. 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 [95, 113, 191]. Additionally, there were no differences in BDNF or NGF levels in peripheral blood mononuclear cells (PBMCs) of patients and controls in the Martinez study [112]. However, Paz et al. reported increased BDNF levels in the cerebellar cortex of schizophrenic patients [135], while Yang and colleagues displayed that the levels of proBDNF and BDNF pro-peptide in the cerebellum of patients with schizophrenic were lower than the control group. In their study, mature BDNF and BDNF pro-peptide production in the brain and liver were abnormal, suggesting that the brain–liver axis has a role in psychiatric disorders pathophysiology [193].

In newly diagnosed psychosis patients, serum NGF levels decrease, and this may be a promising biomarker in the diagnosis or screening for patients with schizophrenia [130, 189, 190, 194]. A synaptic plasticity defect observed in schizophrenia may be associated with NGF and its receptor (NGFR). A positive association between schizophrenia and the NGF rs6330 and the NGFR rs11466155 and rs2072446 SNPs was reported [194]. Alterations of neurotrophins in an animal model of schizophrenia have been confirmed. In animals injected subchronically with ketamine (Ket), which is an excellent 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 the cerebellum [15]. 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 [6].

BDNF and NGF induce a neuropeptide precursor called VGF nerve growth factor inducible (VGF). VGF is involved in cerebellum granule cell development. In mice that VGF was overexpressed, some schizophrenia-like behaviors and motor disabilities were observed [121].

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

CND	Study model	NF/R	Effect	References
ADHD	Human case-control	BDNF	Plasma protein level increased in child patients	Shim et al. [164] and Shim et al. [165]
		BDNF	Serum protein level unchanged in child patients	Scassellati et al. [155]
		BDNF	Serum protein decreased in adult Caucasians	Corominas-Roso et al. [35]
		NGF, BDNF	No significant changes in the Turkish population	[21])
		GDNF, NT-3	Serum proteins level were higher in the Turkish population	
		NGF	Serum protein level increased in child patients	Guney et al. [67]
ASDs	Rat-infected Borna disease virus	NGF, BDNF and NT-3, and their respective Trk receptors	Unchanged in the cerebellum	Zocher et al. [198]
	Human case-control	NT-3, NT4/5	Decreased in the spot-blood of newborns	Nelson et al. [124]
	Postmortem human case-control	NT-3	Increased in cerebellar samples	[148, 149])
	Mouse model of Rett syndrome	NGF	Decreased or unchanged on the cerebellum	Schaevitz et al. [156]
	Human case-control Rett syndrome	NGF	Decreased with age	Calamandrei et al. [24]
Congenital ataxia	Mouse model	BDNF	Decreased mRNA level in the granule cell layer	Qiao et al. [138]
		NGF	Unchanged	
Ethanol neurotoxicity	Rat cerebellar vermis	TrkA, TrkB, TrkC	Decreased	Moore et al. [122]
	Neonatal rat cerebellar granule cells	BDNF, NT-3	Decreased secretion	Heaton et al. [75]

(continued)

Table 1 (continued)

CND	Study model	NF/R	Effect	References
	Neonatal rat cerebellum	BDNF, NGF, TrkA, TrkB, TrkC, and p75NTR	Decreased expression	Dohrman et al. [43], Heaton et al. [73], Light et al. [100], Light et al. [101, 102] and Moore et al. [123]
	Granule cells	BDNF	Inhibit its activation effect on the ERK pathway	Ohrtman et al. [127]
	The cerebellum of short-term ethanol exposed mouse	NGF, TrkA	Increased mRNA and protein level	Wang et al. [186]
		BDNF		
		TrkB, p75NTR	Unchanged	
	Explant culture of rat cerebellum	GDNF	Decreased release despite unchanged mRNA expression	McAlhany et al. (1999)
	Cerebellar granule cells	GDNF	Ethanol inhibited its activation effect on the SFK-Cas-ERK1/2 pathway to promote axonal outgrowth	[28])
Medulloblastoma	MB cells	NGF, TrkA	Suppressed their proliferation	[8])
		NGF, TrkA	Induced apoptosis	Chou et al. [31] and Li et al. [99]
	MB patients	TrkA		Ohta et al. [128]
	Whole-genome microarray on MB tumors	TGF-ß	Influence progression and metastases	[9])
	MB transgenic mouse	TGF-ß	Obstruction of TGF-ß leads to restriction of MB	Gate et al. [57]
Schizophrenia	Human case-control	NGF	Plasma protein level decreased	Lee and Kim [95] and Xiong et al. [190]
		NGF, BDNF, TrkA,	Unchanged in PBMCs	Martinez-Cengotitabengoa et al. [112]
		TrkB	Differential expression of its different isoforms in PBMCs	Martinez-Cengotitabengoa et al. [112]

(continued)

Table 1 (continued)

CND	Study model	NF/R	Effect	References
		BDNF	Unchanged in the cerebellar cortex	Paz et al. [135]

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.

Neuregulin-3 (Nrg3) is another growth factor that is considered a risk factor for schizophrenia. Its mRNA and protein are expressed in embryonic and postnatal periods. Apart from different brain parts, it is found in Purkinje cells and granule gneurons of the cerebellum [142].

Williams Syndrome

Williams syndrome (WS) is a rare neurodevelopmental disorder that affects 2–5/100,000 people [4], and a 1.6 Mb deletion on chromosome 7 (7q11.23) causes it [14]. This syndrome is characterized by an enlarged cerebellum, mild-to-moderate mental retardation with a deficit in visuospatial processing, and an oversensitivity to sound [14]. NGF levels in the serum of WS patients are higher than in normal people, and they remain continuously higher during childhood. This is in contrast to normal people, who have a higher serum NGF only in early childhood [23].

Other Cerebellar Neurodevelopmental Disorders

There are some other disorders of the cerebellum, such as Joubert syndrome [76, 106, 131, 197], Dandy–Walker malformation [10, 53, 68, 118], pontocerebellar hypoplasia [118, 184], cerebellar vermis hypoplasia [118], and developmental dyslexia [168, 169] that occur during development. To our knowledge, there is no data available about any association of neurotrophic factors with these conditions, which suggests new areas of research.

Nucleosome remodeling and deacetylase (NuRD) have an essential role in cerebellar plasticity and neural development. Variations in NuRD's subunits are considered crucial risk factors for neurodevelopmental and psychiatric disorders [reviewed in [78]]. Epigenetic mechanisms such as methylation, histone modification, and miRNA also impact normal and abnormal development of the cerebellum [reviewed

in [161]]. The role of neurotrophic factors on cerebellar neurodevelopmental disorders is summarized in Table 1.

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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 various stages of development. Among these mechanisms, apoptosis, autophagy, and unfolded protein response play critical roles in regulating development by affecting cell fate. All of these pathways are involved in the regulation of cell numbers via determining the life and death cycles of the cells. In this chapter, we first explain the brief mechanisms that are involved in the regulation of apoptosis, autophagy, and unfolded protein response, and later, we briefly describe how these mechanisms play roles in general development. We next address the critical role of these pathways in cerebellar development regulation and how they will aid in our knowledge of the processes behind neurodevelopmental disorders. Additionally, we summarize the present findings on neurological symptoms and disorders related to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and their linkage to autophagy pathways in the cerebellum.

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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
CARDs	Caspase recruitment domains

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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 A2
IGL	Internal granule layer
IGL	Internal granule layer
IRE1	Inositol-requiring enzyme 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

TGFs	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 critical 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 other 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 the 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 the 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 (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 [86, 106]. Apoptosis is a genetically conserved pathway in all metazoans, such as in nematodes, insects, and mammals [36, 80, 137]. During the early process of this type of cell suicide, cellular content is condensed, and cell shrinkage is observed. 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 [44, 106, 135] (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 [36, 44, 79, 80] (Fig. 1).

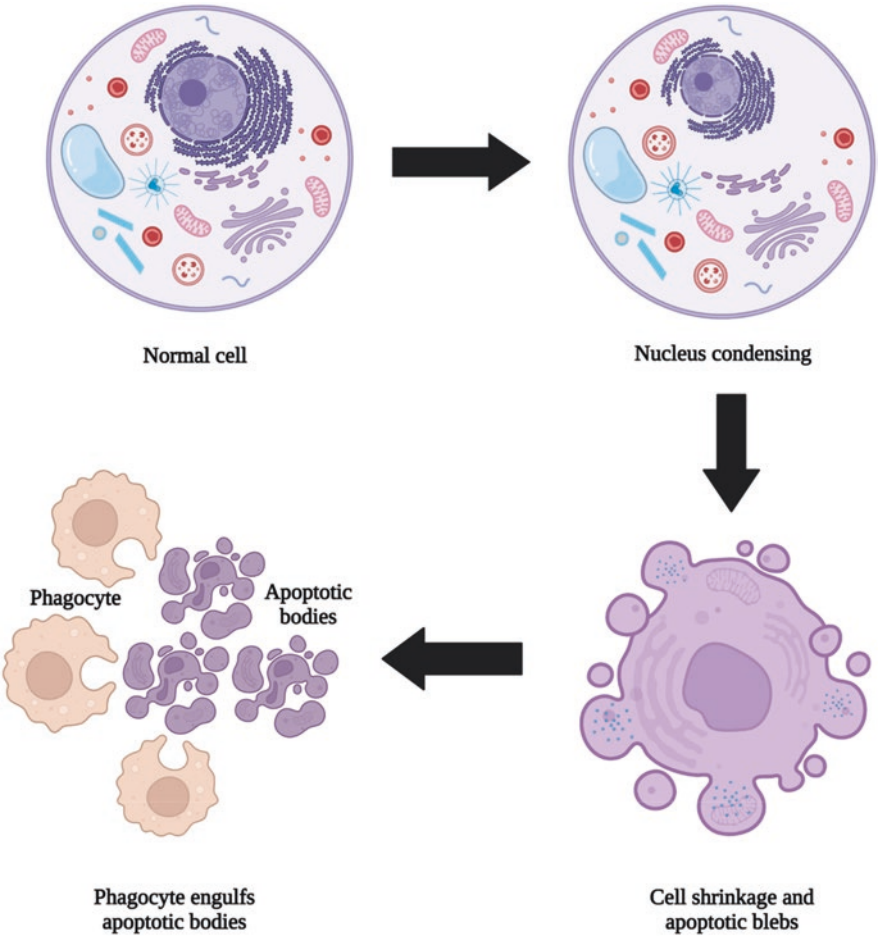

Fig. 1 Cellular morphology changes during apoptosis. Apoptosis is an ATP-dependent mechanism that includes chromatin condensation (pyknosis), inter-nucleosomal DNA fragmentation, membrane blebbing and budding, and finally the formation of small membrane-bound vesicles, called apoptotic bodies

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) [35]. 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) [54, 65, 129]. To date, several 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. Proapoptotic caspases are divided into the initiator procaspase group (i.e., procaspase-2, -8, -9, and -10) and into the effector (executive) procaspase group (i.e., procaspases-3, -6, and -7) [54, 129]. Following activation, the executive caspases degrade most vital proteins in the cells, disrupting 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; acts as a nucleus for actin depolymerizing enzyme), and poly (ADP-ribose) polymerase (PARP) are targeted proteins that are cleaved during apoptosis [25]. In addition, caspase-activated DNase (CAD) that mediates DNA ladder hallmarks of apoptosis is activated by caspase-3 and -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 subunits followed by assembly of active heterotetramers [36, 129] (Fig. 2).

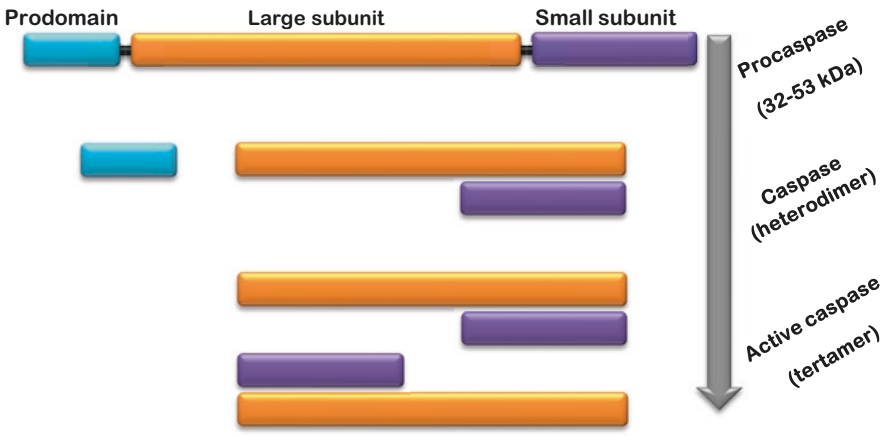

Fig. 2 Structure and activation of caspases. Inactive form of caspases (procaspases) includes three subunits. The mechanism of caspase activation is initiated by the autoproteolysis of Asp residues into large subunits, followed by the assembly of active heterotetramers. Following proteolysis cleavage,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

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 leading executive caspase that is activated by any of the initiator caspases (caspase-8, -9, or -10) [30, 36, 44, 54, 80, 106, 129].

Cell Death Receptor Pathway

The extrinsic pathway is mediated by the activation of death receptors, which are transmembrane receptors that transmit apoptotic signals from the cell surface to the intracellular signaling pathways via receptor–ligand interactions [67, 101, 129]. Death receptors involve Fas (CD95) and TNF receptor (TNFR1) as well as TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5 (TRAIL receptor 1 and 2, TRAIL-R1 and -R2) [66]. 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 [25, 36, 80] (Fig. 3). In this scenario, the first step is trimerization and clustering of receptors by related ligands. Upon ligand binding, cytoplasmic adaptor proteins are recruited via intracellular receptor death domain (DD), 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 domain (DED), thereby forming the death-inducing signaling complex (DISC) [36, 44, 80, 129]. An increase in the local concentration of procaspase-8 at the DISC results in their autocatalytic activation (Fig. 3). Activated caspase-8 finally cleaves and activates the effector caspase-3, leading to 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 cells that require the contribution of a mitochondrial pathway to achieve the apoptotic process are classified as type II cells [131]. In type II cells, the receptor-mediated signaling is not strong enough to activate caspase for the execution of apoptosis, so the signal needs to be amplified via mitochondria-dependent apoptotic pathways [36, 52, 54, 80, 129, 153]. The link between the cell death receptor pathway and the mitochondria is provided by Bcl-2 family member Bid. Bid is cleaved by caspase-8 and in its truncated form (tBID) translocates into 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 [36, 80, 106, 129].

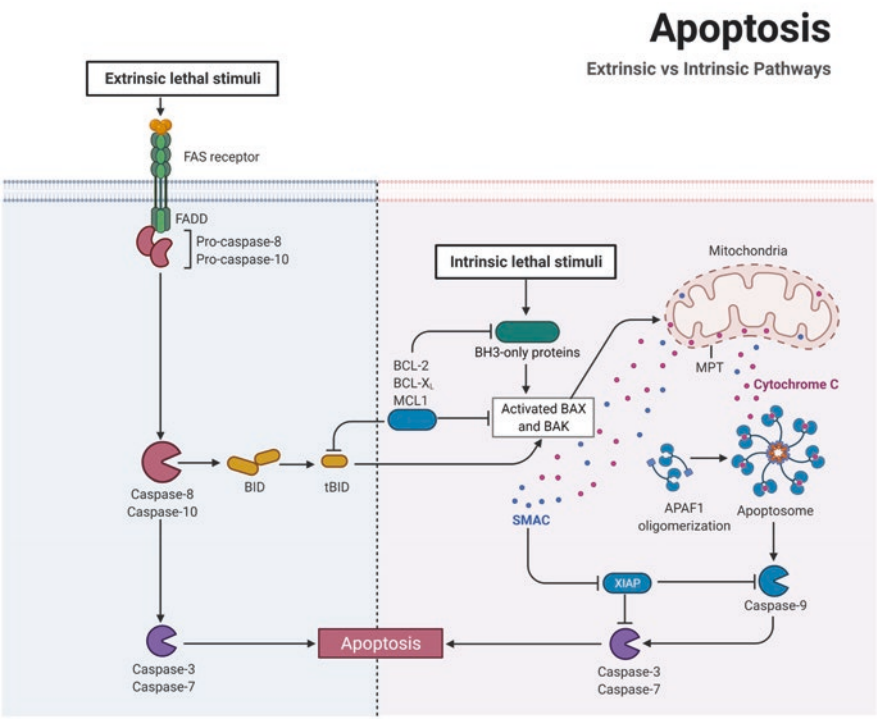

Fig. 3 Intrinsic and extrinsic apoptosis pathway. Extrinsic pathway (1) is commenced via death receptors, which later activate initiator caspases (like caspase-8) with subsequent Bid protein truncation (link to mitochondria) or directly activates caspase-3, and -7 (executor caspases) and induces nuclear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

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 [19, 44, 54, 129, 153]. Cellular stresses cause an increase in the permeability of the outer mitochondrial membrane and opening of the mitochondrial permeability transition (MPT) pore, which is controlled by members of the Bcl-2 family proteins [36, 80, 129]. 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 [57, 102, 111]. 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) [127, 153]. 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) [102, 111] (Fig. 3). In general, oligomers of Bak, Bax, and Bok can form channels by themselves to induce permeability membrane transition (PMT) [9]. 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 [9, 102]. Puma and Noxa are also involved in *p53*-mediated apoptosis [26, 129]. Bcl-2 family proteins control release of the mitochondrial proteins cytochrome c (cyt c), the second mitochondria-derived activator of caspase (Smac)/direct IAP binding protein with low pI (DIABLO), and Omi/high-temperature requirement protein A2 (HtrA2) into the cytoplasm [9, 11, 129]. 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, 11, 36, 129] (Fig. 3). This type of procaspase-9 clustering leads to caspase-9 activation, which subsequently activates downstream executive caspases such as caspase-3, -7, and -6 and ultimately leads to apoptosis. Smac/DIABLO and the serine protease HtrA2/Omi promote apoptosis by inhibiting inhibitors of apoptosis proteins (IAPs) activity [11, 55, 107]. 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 [36, 80, 129, 143] (Fig. 3). Almost all of the morphological and biochemical features of apoptosis are mediated through the activity of caspases [7, 25].

Apoptosis in Development

Apoptosis literally means “falling off” (as leaves drop from trees) in Greek, and this analogy suggests that cell death is necessary for the life cycle of organisms [46, 60, 87, 106]. An example of the impact of PCD on development is seen in lymphocytes. Most lymphocytes die via apoptosis due to negative selection or genetic rearrangement, thereby verifying the constant cellular pool of functional immune cells and lymphocyte numbers [128]. Moreover, apoptosis is critical for the development of reproductive organs [115]. Apoptotic processes are widely involved in the regulation of proliferation, differentiation, development, and tissue homeostasis [42]. Additionally, it has been shown that inhibition of caspase-8 decreased neurological impairments and edema, enhanced cell proliferation, and neurofilament levels in the damaged cerebrum [134]. Furthermore, the study’s findings indicated that the death receptor pathway may be a critical target for long-term therapy of cerebral ischemia–reperfusion, since it not only activates downstream cleaved caspase-3 but also activates the mitochondrial route through Bid [162].

Autophagy and Its Role During Development

Autophagy

Autophagy is a tightly regulated catabolic process used by eukaryotes for recycling and degrading organelles, proteins, and other cytoplasmic components in a lysosomal-dependent manner. Autophagy occurs in three typical forms, including microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA) (Fig. 4) [20, 89, 113, 125]. Microautophagy and CMA are directly mediated by lysosomes to immediately degrade small cytosolic portions or chaperone-associated molecules, respectively [14]. 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 [41, 53, 89, 104, 117] (Fig. 5). Autophagosomes are then fused with lysosomes, and this forms the autophagolysosome (Fig. 5). 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 [5, 157].

The molecular components of this pathway were first discovered in the yeast *Saccharomyces cerevisiae* and included autophagy-related proteins (ATGs) [121]. Most autophagy stimuli converge at the phosphatidylinositol 3-kinase

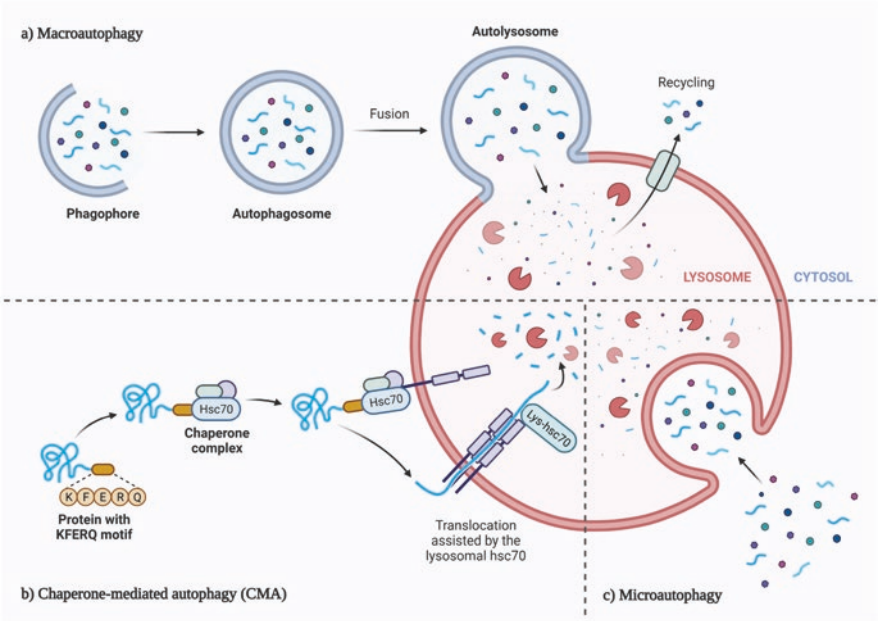

Fig. 4 The machinery of autophagy. Macroautophagy (**a**), chaperone-Mediated autophagy (**b**), and microautophagy (**c**) are three types of autophagy

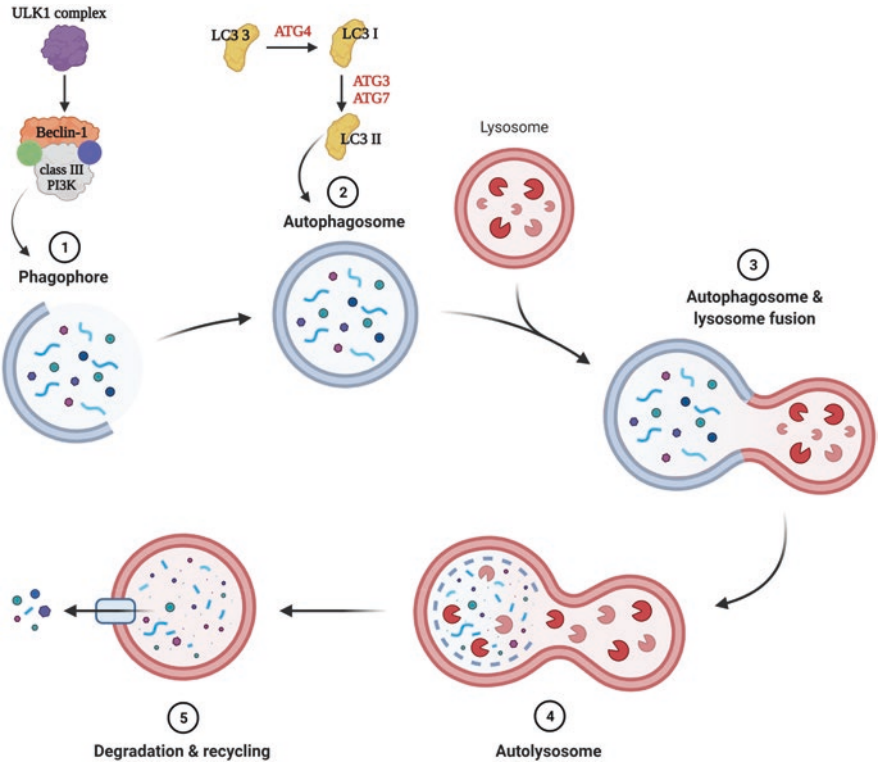

Fig. 5 Schematic representation of autophagy pathway. Autophagy is a process for the degradation and recycling of cellular compartments in lysosomes, including phagophore, autophagosome, and autolysosome

(PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway that is the best-characterized modulator of autophagy in most cells [17, 61, 89] (Fig. 6). This signaling pathway plays a vital role in multiple cellular functions such as proliferation, adhesion, migration, survival, and invasion [6, 73, 85, 154]. The PI3K-Akt-mTOR pathway integrates signals from growth factors, energy, and nutrients to adjust proliferation and cell growth through various cellular mechanisms [39, 56, 105, 154]. The serine–threonine protein kinase Akt, also called protein kinase B, is upstream of mTOR and the downstream effector of PI3K. Inactivation of mTOR complex 1 by starvation conditions activates Unc-51-like kinase (ULK), which initiates the autophagy process; however, under normal nutrient conditions, mTOR complex 1 phosphorylates ULK1/2 and Atg13 to inhibit the initiation of the autophagy pathway [130, 142, 154]. Therefore, the core machinery for the initiation stage during autophagy induction is the ULK1/2 complex, consisting of ULK, Atg13, and FIP200. Upon autophagy initiation, a complex nucleation arises when the PI3K complex binds to its core units, such as Beclin 1 (the human orthologue of murine Atg6) [40, 103, 104]. This complex resides on the isolated membrane and facilitates the recruitment of other ATGs to the unit (Fig. 5). During autophagosome

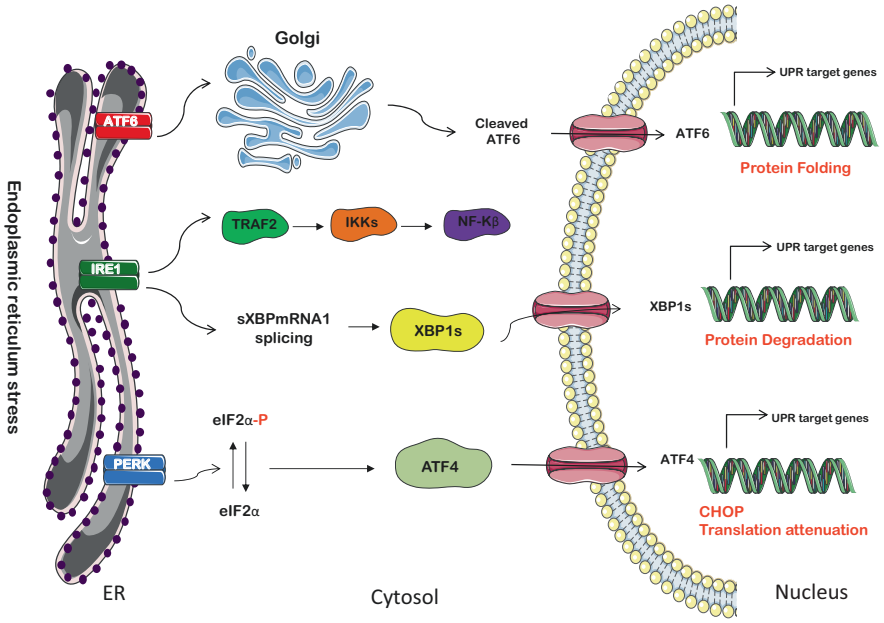

Fig. 6 Schematic representation of ER stress and unfolded protein response. ER stress activates UPR proteins (e.g., PERK, ATF6, and IRE1) in the endoplasmic reticulum. Briefly, activated PERK promotes ATF4 activation via phosphorylation of eIF2α. Activation of the IRE1 arm of UPR induces XBP mRNA splicing in the cytoplasm, subsequently leading to the activation of UPR target genes. The ATF6 arm is initiated via cleavage of ATF6 in the Golgi, which is later targeted to the nucleus and induces the expression of UPR-responsive genes

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 LC3I. Phosphatidylethanolamine (PE) is then conjugated to LC3I by Atg7 and Atg3, and it creates LC3-II that can stably insert into the autophagosomal membrane [51, 53, 58, 92, 117].

Role of Autophagy in Development

There is emerging evidence that autophagy plays a critical role 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 [18]. Additionally, autophagy is essential for survival during neonatal starvation and cell differentiation during lymphopoiesis, erythropoiesis, osteogenesis, and adipogenesis [1, 18, 116]. The Atg7-deficient mice showed severe anemia because there was a lack of sufficient erythropoiesis. It has been suggested that

erythroid differentiation depends on autophagy for mitochondria removal [119]. Adipocytes mainly harbor lipid droplets that have been identified as a substrate for autophagy [34]. In addition to the differentiating functions, autophagy is also crucial for the survival and viability of terminally differentiated cells such as neurons. Autophagy knockout mice showed uneven numbers of neural stem/progenitor cells, resulting in delayed development. The phenotype of Ambra1 and ULK1 mutant mice confirmed the importance of autophagy and plausible mechanisms during the development of the nervous system [43]. Ambra1 is a vertebrate-specific protein highly expressed in the nervous system and positively regulates autophagy by promoting Beclin-1 binding to Vps34 [43]. ULK1 is an ATG protein involved in autophagy initiation, and its deficiency leads to defects in terminal neuronal differentiation and causes abnormal axonal formation in the cerebellar granule neurons [145]. 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, the trans-placental 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 [94]. Confirming these reports, Atg5 null mice are normal at birth, but die within one day after birth, highlighting the importance of autophagy for embryo development [94].

During cerebellum development, the coordinated formation of various neuronal cell types inside the cerebellar primordium is crucial. All GABAergic and glutamatergic neurons are generated from the ventricular zone and rhombic lip of the cerebellum, respectively. Purkinje cells (PCs) and cerebellar nuclei (CN) neurons are some of the first neurons to develop between embryonic days (E) 9 and 13. Prior to embryonic day 14.5, postmitotic and differentiated PCs and CN neurons move to the cerebellar primordium's PC plate (PCP) and nuclear transitional zone (NTZ). Uncertainty exists about the cellular and molecular processes enabling early cerebellar neurogenesis, migration/differentiation, and formation of connections. Macroautophagy is critical for controlling cellular phenotypes, such as epithelial-to-mesenchymal and endothelial-to-mesenchymal transitions. TGF-1 is also involved in modifying cellular phenotype through a variety of processes, including autophagy. It is a critical component of pre- and postnatal development. The study's findings indicated that the canonical TGF-β signaling pathway was activated throughout the temporal frame associated with the creation of the PCP and NTZ.

Additionally, the findings reveal that an active TGF-β signaling pathway may upregulate the expression of N-cadherin and β-catenin sequentially and temporally, with maximal expression at E11/E12 and subsequent elevation of Cdh8 and NCAM expression at E12 and E13. Interestingly, the findings demonstrated that enhanced TGF-β signaling occurs simultaneously with autophagic flux inhibition at E11/E12. However, basal autophagy occurs throughout the E9 to E10 embryonic phases. This study established a critical role for the TGF signaling pathway and its regulatory effects on Cadherin expression and autophagic flux during cerebellar development, all of which may contribute to the proliferation, migration/differentiation, and positioning of CN neurons and PCs within their domains [28].

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 [97]. These proteins are induced by some stress conditions, including glucose starvation, treatment with cellular glycosylation inhibitors, calcium ionophores, or amino-acid analogs [97]. The significant difference between these proteins and heat shock proteins (HSPs) is that GRPs are not induced by increasing temperature [97]. GRP78 was shown to have some activity like immunoglobulin heavy chain binding protein (BiP) [4, 120]. It can also permanently bind to various 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 (for example, 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 [93]. It has also been shown that the highly conserved *GRP* element, which is vital 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 can transactivate the *GRP78* promoter through interaction with this CCAAT motif [156].

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 homolog 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 does not affect 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 [124]. In addition to tunicamycin, other inducers of GRPs include dithiothreitol (DTT) as a sulfhydryl reducing agent, amino acid analogs, severe glucose depletion, and oxygen, which increase the protein flux, decrease Ca^{2+} levels, and disrupt lipid homeostasis to induce UPR in the cells [98, 138, 149, 151].

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, emphasizing its importance in neurodegenerative diseases. It has been shown that UPR signaling modulates neurodegeneration depending on the disease context [29, 70].

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. 6) [139, 149].

The IRE1 pathway is considered to function as a major and the most conserved arm of the UPR from yeast to humans [139, 149]. IRE1α and IRE1β have been known as two homologs of mammalian IRE1. While IRE1α is expressed in all cells and tissues and is a primary mediator of UPR signaling, IRE1β is expressed only in the intestinal epithelium [149]. Activated IRE1α, which shows endoribonuclease activity, cleaves a 26-base fragment from the mRNA encoding the X-box binding protein-1 (XBP1) [100, 164]. 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 [164]. Unspliced-XBP1 mRNA is constitutively translated into XBP1U [3, 118].

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 essential role as a putative ER stress response element (ERSE)-binding protein, introduced in 1998 by Yoshida et al. [163]. 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 a posttranslational mechanism, which is a response to stress [163]. 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 [68, 161]. 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 [164].

ATF6α and ATF6β are two distant homologs of ATF6 but both are ubiquitously expressed in all tissues [144]. 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 [144]. It has been suggested that the relative levels of ATF6α and ATF6 may regulate the strength and duration of ATF6-dependent ERSR gene induction and cell viability. In addition, ATF6α is a strong but labile transcription factor, while ATF6β is a weak and stable transcription factor. A gel shift assay showed that they compete with each other in binding to the GRP78 ERSE [144].

Harding et al. first introduced PERK in the mouse ER in 1999 [63]. 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 homolog of PERK, which is pancreatic eIF-2α kinase (PEK) from the rat pancreas [140]. 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 [63]. 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) [62, 64, 109]. 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 XBP1U induces in response to non-ER stress [118]. Activation of ATF4 and CHOP negatively regulates mTOR via Redd1 expression in response to oxidative and ER stress [82].

To get a better understanding of the potential role of ER stress-associated proteins in cerebellar diseases, researchers evaluated the expression of ER stress sensors and their downstream targets in the postnatal rat developing cerebellar cortex. For the first time, the stress sensors PKR-like endoplasmic reticulum kinase (PERK) and inositol-requiring enzyme 1 (IRE1) were activated in properly growing granule cell (IGL) precursors. In the interior granular layer, a second proliferating pPERK population was also observed. In general, when profiles from early and late postnatal ages were compared, the density of UPR protein-positive cells decreased dramatically [122].

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 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 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 [15].

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 processes, as mentioned earlier, to achieve a high-quality protein suitable for the growth and survival of the cell [12, 77, 132]. In addition to the metabolic enzymes, molecular chaperones, chemical chaperones, and other small molecules affect proteostasis. Several vital processes, including heat shock response (HSR) [71, 83, 150, 152] and UPR [15, 77], 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 heat shock treatment (hyperthermia), while the synthesis of most other proteins decreases [71]. Based on the time and region, there is also a significant difference between the kinetics of various HSPs [71, 72]. Northern blot analysis has indicated that there is differential induction of various classes of HSP mRNAs by ischemia. Within 4 h post-ischemia, the HSP70 family mRNAs were induced and then rapidly decreased, while HSP27 and HSP47 mRNAs were maximally increased at 24 and 48 h post-ischemia, respectively. In addition, in situ hybridization showed that mRNAs of inducible HSP70s were localized in the core region of the infarct 2 h post-ischemia, and at a relatively late period (4–8 h), they moved to the penumbra region [72]. 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 [71].

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) [70]. Although clinical manifestations of these diseases are different, all involve the accumulation of misfolded proteins and are now classified as protein misfolding disorders (PMDs) [70, 141].

In addition to their role in the nervous system and ischemia [59, 70, 74, 148], the role of UPR and proteostasis have also been demonstrated in general development [148], development of B- and T-cells [16, 50], and lens proteins [45, 159].

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 [45]. In the lens epithelium of patients with cataracts (high myopia-related or age-related

cataracts), 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 cataracts, which may be associated with this type of cataractogenesis [159]. 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 [110].

Apoptosis and Cerebellum Development

In the cerebellum, ventricular zone (VZ) and rhombic lip (RL) are the sources of all cerebellar cell types. The VZ and the external granule layer (EGL) are able to proliferate and migrate to the granule layer (GL) and sub-ventricular zone. PCs 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 [8, 22].

Apoptosis of Stellate and Basket Cells

Progenitor cells such as stellate and basket cells are produced from the ventricular zone (VZ). 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 [108].

Apoptosis of Purkinje Cells

PCs migrate from the VZ and are placed between the molecular layer and GL cells, forming a single-cellular layer [22]. They are key cells in the cerebellum that are targeted in many neurological mutations in mouse models to study PC death. Two periods of apoptosis occur in these cells: the first is the embryonic period and the second is the postnatal term. Regulation of PC apoptosis occurs through the connection to climbing fibers. First, one climbing fiber interacts with several PCs during the first postnatal week in rats. Then, the climbing fibers fix their final connection.

The PCs, which cannot react to climbing fiber, undergo apoptosis and are deleted. However, the apoptotic PCs can interact with climbing fibers, and this causes them to retract. Thus, the connection between PCs and climbing fibers occurs on a one-to-one basis [22]. There are also many genes that interfere with the regulation of PC death. Two mouse models, Toppler and Woozy, are mutants in which PCD is observed in PCs by apoptotic pathways and sometimes with activation of autophagic mechanisms. This cell death may be different from formal PCD. Many of PCs will die during normal ageing, using mechanisms similar to apoptosis [108].

The purpose of the study was to provide experimental evidence about nervous system damage by measuring PC number as well as their apoptosis in rat cerebellum after fatal and nonfatal electric current injury utilizing histological and immunohistochemical analysis. Cerebellar PCs are one of the biggest neurons in the nervous system, with many branching extensions, which makes any abnormal alterations readily visible. Additionally, because of their low resistance, PCs are one of the most impacted sections of the central nervous system (CNS) by electrical currency. The study's findings indicated that apoptosis and PC loss were involved in the pathogenesis of the immediate and long-term effects of the electrical injury on PCs, which will aid forensic pathologists in determining the cause of death, residual damage, and disability following electric shock [84]. Numerous studies have shown that UPR has a role in frontotemporal dementia (C9-FTD). UPR indicators are closely related to granulovacuolar degeneration (GVD) in human neuropathology. The surveys indicate that UPR indicators are enhanced in C9-FTD and are related to dipeptide pathology and GVD. Increased expression of UPR markers and casein kinase 1 delta (CK1δ) in cerebellar and hippocampal granule cells may be a specific hallmark of C9-FT [48]. Intriguingly, it was shown that activating the UPR modifies glutamate neurotransmission, particularly in the cerebellum of a mouse model of autism. The results demonstrate that the R451C autism-linked mutation in neuroligin 3 induces UPR in vivo, which seems to result in changes in synaptic function in the cerebellum of a mouse model harboring the R451C autism-linked mutation [146].

In humans, it has been shown that cerebellar PC death occurs in essential tremor, ataxia, and a variety of other neurodegenerative diseases. Shaker mutant rats have an X-linked recessive mutation that results in hereditary degeneration of cerebellar PCs that are "at risk." This deficiency may emerge postnatally in the shaker mutant rat's confined anterior (ADC) and posterior (PDC) vermal degeneration compartments between 7 and 14 weeks of age as a natural phenotype. The ultrastructural analysis reveals that "at risk" PCs perish as a consequence of autophagic activation-induced apoptosis. Furthermore, our observations imply that both apoptosis and autophagy must be suppressed concurrently to prevent the death of "at risk" PCs [37, 38].

Apoptosis of Granule Cells

Granule cells migrate from the EGL to the GL of the cerebellum. The apoptotic cells in GL have been verified, and it was shown that they are postmitotic neural cells that could not produce a proper synaptic connection with PCs in the molecular

layer [22]. During the first week of postnatal life, there is an established cell loss within the granule cell layer. Many granule neurons in both mitotic and postmitotic regions of the EGL undergo DNA fragmentation [155]. 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 granule cells, first extensively proliferate, and some of them start differentiating into mature granule cells [31]. The first in vitro apoptosis model for the CNS was recently identified. It was shown that cerebellar granule cells undergo apoptosis when deprived of depolarizing extracellular potassium levels [27]. Additionally, the in vivo correlation between apoptosis in cerebellar granule cells has been recently reported by Wood et al. [155], which shows DNA fragmentation in the granular layer of the newborn rat's cerebellum. Cerebellar granule neurons are a perfect model system to study neuronal apoptosis because they live and survive for weeks when they are maintained in depolarizing potassium concentrations. 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 [47]. To comply with the existing hypothesis that during development, transforming growth factors (TGF-β) might play a role in the 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, -β2, and -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 [31].

Additionally, there is evidence of a p53-independent apoptotic pathway for the 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 those in wild-type mice (14). It has been previously suggested that the 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 [158].

Apoptotic cells are identified as immature granule cells and/or their GCPs. Analysis of apoptotic pathways has indicated that caspase-3 and -9 are expressed in cerebellar germinal zones and activation of caspase-3 is essential 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 granule cells from death. The experiments showed that caspase-3 is not activated during apoptosis of GCPs/pre-migratory granule cells. Therefore, early neuronal death of GCPs/pre-migratory granule cells may be caspase-3-independent. The naturally occurring neuronal death (NOND) of GCPs/pre-migratory granule cells is possibly related to the establishment of the correct ratio between granule cells and PCs. The vast cell death in

EGL neurons is related to folia formation during the 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 granule cells 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 granule layer (GL). Therefore, this PCD is different from early NOND and is caspase-dependent [108].

Apoptosis of Cerebellar Nuclei Neurons

After treating neonatal rats with ethanol, cerebellar nuclei neurons undergo apoptosis. The axotomy is initiated in cerebellar nuclei 3 h after lesion formation, and neurodegeneration begins within 48 h. Apoptotic cell morphology has been observed, but during the normal development of cerebellar nuclear neurons, apoptosis does not occur [108].

Autophagy and Cerebellum Development

As discussed above, autophagy is a self-degradation lysosomal system initially described in single-cell organisms as an adaptation mechanism not only to nutrient supply fluctuations but also to recycle various cellular organelles [113, 160].

The nervous system complex ontogenesis is especially sensitive to the 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, 13, 103]. Autophagy plays an essential role in the late stages of embryonic and postnatal development [32]. Defects in autophagy lead to impairment in the number of neural progenitors, thus causing incorrect differentiation and development [1]. For example, an autophagy malfunction causes inappropriate neurotransmitter processing and secretion [1]. Many neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) are caused by defective autophagy mechanisms [90, 91]. The other consequences of defects in autophagy genes such as Atg5 and Atg7 mutant mice include perinatal lethality in suckling kids and neurodegeneration symptoms [90, 91]. In addition, ablation of Atg7 causes dystrophy of PC axon terminals in the cerebellar nuclei [32, 112].

Another study on the *ULK1* gene in mammals, which is an orthologue of the yeast *Atg1*, showed its essential role in autophagy machinery. ULK1 is a protein kinase that plays a significant role in early autophagosome formation. When autophagy is induced, ULK1 kinase separates the Ambra1/Beclin 1 complex from the

dynein complex to initiate the autophagy process. Ambra1 is expressed in the CNS during embryogenesis, specifically in the neural plate. In mice with the *Ambra1* mutant, the autophagy machinery is deficient, and apoptosis was observed. This result showed the relationship between autophagy, apoptosis, and cell proliferation. Therefore, Ambra1 is an essential protein for controlling cell proliferation during the development of the CNS [32]. These data indicate that autophagy is involved as a part of PCD in parallel with apoptosis in the cerebellum. The autophagic cell death act as an alternative PCD when apoptosis is inhibited in the rat cerebellar granule cell [114]. Both reactive oxygen species (ROS) and autophagy promote apoptosis in this model [114]. Degeneration of PCs 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 PC degeneration (*pcd*) in mice. This highlights a link between mitochondrial dysfunction, autophagy, and PC degeneration in the cerebellum [21]. In conclusion, autophagy is an essential process for the survival and development of cerebellar cells [112].

COVID-19, Cerebellum, and Autophagy

Neurological problems associated with the recently identified severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are becoming more prevalent [165]. Since the pandemic outbreak of the coronavirus disease 2019 (COVID-19), which was triggered by the SARS-CoV-2 infection, a variety of neurological manifestations have been reported, ranging from headache, dizziness, and seizures to encephalopathy, meningitis/encephalitis, and stroke. It was shown that acute necrotizing encephalopathy with a predominance of cerebellar involvement and cognitive impairment might be one of COVID-19's neurological symptoms [24]. While the cytokine storm seems to be the primary cause of mortality, the mechanism by which SARS-CoV-2 generates enormous amounts of cytokines remains unknown. Two separate investigations on the SARS-CoV-2 protein ORF3a revealed that, although this protein induces autophagosomes, their maturation is ultimately hindered, resulting in autophagy failure and increased inflammation, a trait that seems to be specific to SARS-CoV-2 [96]. Indeed, a significant correlation between the uncontrolled inflammation induced by SARS-CoV-2 and autophagy abnormalities has been established [49], suggesting that heightened cytokine storm may be the consequence of autophagy's inability to regulate homeostasis. However, it is unknown if autophagy plays a role in SARS-CoV-2 protein degradation and whether SARS-CoV-2 evades detection and breakdown by blocking A-L fusion during the latter stages of infection associated with cytokine storm. Notably, the inhibition of the A-L fusion seems to be specific to SARS-CoV-2 since a comparable ORF3a protein from SARS-CoV did not inhibit it [96] (Fig. 7).

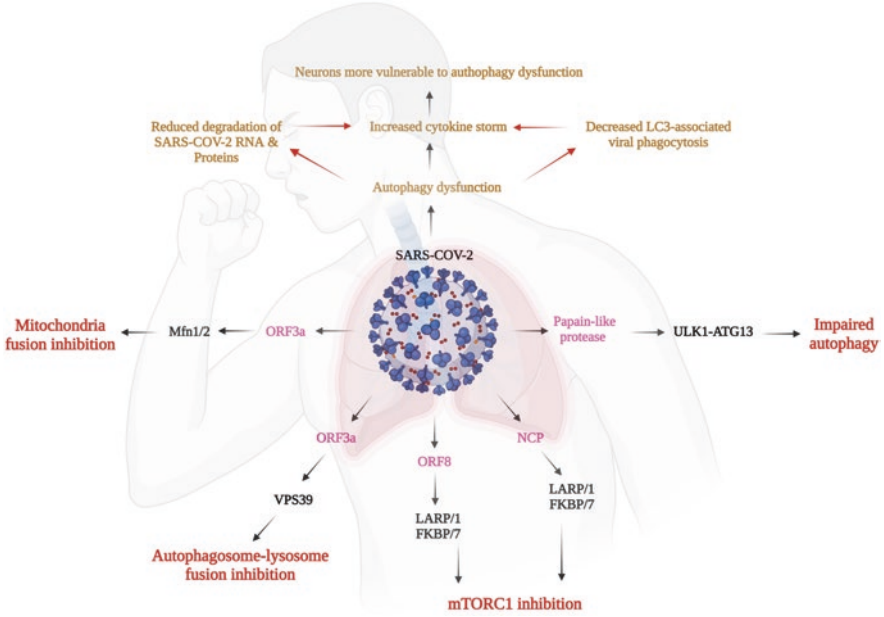

Fig. 7 The molecular mechanisms behind SARS-CoV-2-induced autophagy dysregulation. By attaching to the host protein mitofusin (Mfn) 1 and 2, the SARS-CoV-2-derived open-reading frame (ORF) protein 3a (ORF3a) may block mitochondrial fusion. ORF3a may also impede the fusion of the autophagosome and the lysosome (A-L) by binding to the host protein Vamp6 (VPS39) and the HOPS complex. ORF8 and nucleocapsid protein (NCP) are two additional SARS-CoV-2-derived proteins that may bind to La ribonucleoprotein 1, translational regulator (LARP1), and FKBP prolyl isomerase 7 (FKBP7), respectively, and inhibit mammalian target of rapamycin complex 1 (mTORC1), activating autophagy. By attaching to the Unc-51-like autophagy activating kinase (ULK1) and autophagy-related 13 protein (ATG13) complex, the papain-like protease may block autophagy. Autophagy malfunction caused by SARS-CoV-2 may trigger a large cytokine storm, resulting in the death of susceptible cells such as neurons and microglia [96, 136]

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 the pathogenesis of various neurological disorders [69, 126]. The developing brain is susceptible to different kinds of environmental stresses (e.g., infectious pathogens, pollutants, alcohol, drugs, and malnutrition), which often cause ER stress [126]. 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 [76]. In this condition, the UPR can degrade misfolded/unfolded proteins by activating the ER-associated degradation to ensure a balance of protein folding capacity that is crucial for cerebellar Purkinje cell

survival [75, 99, 166]. 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 indicates that the UPR is developmentally involved during neurogenesis [147]. The UPR is generally responsible for the hierarchy and lineage relationships developed in the CNS cells in various animal models [59, 95]. 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 [23]. Laguesse and coworkers proposed a model suggesting that dynamic regulation of the UPR pathways is critical to switch from direct to indirect neurogenesis [95]. During cerebral cortex development, the UPR promotes neurogenesis. In the rat cerebellum, the development of white matter tracts depends on a dramatic increase in membrane protein and lipid production in oligodendrocytes to facilitate myelin production [123]. A substantial peak in ER stress signals IRE1 and ATF6, but not PERK. In addition, the UPR mediators GRP78, GRP94, calreticulin, CHOP, and PDI have been observed in the developing rat cerebellum [123]. Furthermore, 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 [78]. A few specific ER chaperones can also play a direct role during the proliferation and early development of the cerebellum to ensure homeostasis for increased activity of protein secretion [33]. For example, the ER-resident protein ORP150/HSP12A is involved in cerebellum development. Transgenic expression of this protein in neurons reduces PCs' apoptotic death and their vulnerability to hypoxic and excitotoxic stress, which subsequently leads to maintaining the survival of these cells during cerebellar development [88]. Defects in BAP (SIL1), another regulator of UPR, can also cause damage in PCs [166, 167] and cerebral ataxia disease [10, 133]. The Marinesco–Sjögren syndrome characterized by cerebellar ataxia is associated with mutation in the SIL1 gene [10, 133]. These reports suggest that dynamic regulation of the UPR is needed for balance between proliferation and differentiation in the cerebellum and other tissues [95].

CLCC1, a transmembrane protein in ER, was shown to play an essential role in the maintenance of ER homeostasis in the young cerebellum. Mutation in this gene results in a few pyknotic granule cells in the 3-month-old cerebellum. Bip upregulation and ubiquitin-positive inclusions were observed in these neurons [81].

Conclusion

The role of apoptosis has been investigated in different aspects of development, including cerebellar development. Many vital roles of apoptosis have been identified in the regulation of cerebellar development. Recently, autophagy and the UPR, which are major cellular responses to intra- and extracellular stress, have also played essential roles in regulating cerebellar development. All of the mechanisms described in this chapter are tightly interconnected and affect each other. Therefore,

future research should consider regulating different organ development, including cerebellar development, focusing on the regulatory effects of apoptosis, autophagy, and UPR on this process. As 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 misfunctional organelles, including mitochondria, lysosomes, and the ER, will be a significant asset to increase knowledge in the field of neurodevelopment. Early cerebellar development could be potentially regulated via TGF superfamily and its interaction via the autophagy pathway. This could have potential application for targeting diseases with the early development origin like "Autism," and our future investigation will shed light on this important topic in cerebellar development.

Some neurological disorders might be initiated via SARs-CoV-2 infection and the interaction of the viral protein with autophagy machinery. It has been proven that some of the viral proteins could be detected in patients a few months after recovery [61] and inhibit autophagy machinery. Therefore, it is very important that future investigations address the potential impact of SARS-CoV-2 infection in the early development of the cerebellum via placental transport of viral protein to the fetus and its effect on autophagy. This could be one of the big challenges in developmental biology to address it as post COVID syndrome long-term effects.

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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 the 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 in the Atxn3 gene, which codes for a deubiquitinase enzyme. Defects in the expression of a variety of ubiquitin ligases are associated with Freidrich'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 that restores protein homeostasis in various cerebellar diseases.

Keywords Ubiquitin proteasome system · Spinocerebellar ataxia · Machado-Joseph disease · Medulloblastoma · APC/c complex

Abbreviations

AIP	Atrophin interacting protein
AIUP	Ataxin-1 ubiquitin-like interacting protein
APC/C	Anaphase promoting complex/cyclosome
ASD	Autism spectrum disorder
AT	Ataxia telangiectasia

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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
DUB	Deubiquitinase
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
EI	Ubiquitin-activating enzyme
FRDA	Freidrich'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 mechanisms for rapidly controlling proteins that are required for a short, or defined, period of time. The subunits and assembly of the proteasome have 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 a lack of coordination of muscle groups leads to abnormal gait. Such neurological conditions are often associated with the degeneration of parts of the cerebellum and the 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 a therapy for polyQ diseases [15].

In a mouse model of SCA1 gene expression in the cerebellum of the ATXN1, polyQ mice were compared with wild type and 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 spinocerebellar diseases. It was named after two individuals who first described it [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 the 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 the 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 the 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 that completely deubiquitinates its substrate [28, 34, 35]. According to Windborn et al., it binds to both Lys [48] and Lys [63] ubiquitin linkages but preferentially cleaves Lys [63] 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, 36]. 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 [37, 38]. Its cellular role has been related to protein quality control [38]. Chai et al. [39] 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 [40, 41]. These models will contribute to determining the role of ataxin-3 and its polyglutamine form in the development and treatment of SCA3/MJD.

Spinocerebellar Ataxia 5 (SCA5)

Mutations in the *SPTBN2* gene reportedly cause SCA5 [42]. 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 [43]. The alpha chain is reported to have E2 ubiquitin conjugase activity as well as E3 ubiquitin ligase activity [44–46]. 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 [47]. CACNA1A, also known as SCA6, is associated with the E3 ubiquitin ligase BCL6 [48]. 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 the transcription of a number of genes [49, 50]. The DUB protein that contributes to this complex is USP22 [51]. The polyglutamine expansion of the ataxin-7 protein apparently interferes with the function of the USP22 as a gene silencer [51, 52].

Spinocerebellar Ataxia 8 (SCA8)

SCA8 is associated with a trinucleotide repeat expansion in the two overlapping genes *ATXN8* and *ATXN8OS* [53]. The latter codes for an antisense RNA for the ubiquitin ligase KLHL1 [54]. 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 [55]. 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 [56]. 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 [57], 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 [58, 59], 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 the gene for a potassium channel component, KCND3 [60]. This protein has been classified as an E3 ubiquitin ligase of the BTB family in a supplemental table of a recent publication [61].

CHIP and Gordon Homes 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 [62, 63]. In Gordon Holmes syndrome (ataxia associated with hypogonadism), mutations in the *Stub1* gene and loss of CHIP have been identified as the probable cause of this disorder [64]. Ronnebaum et al. [65] concluded that CHIP is required for the 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].

Friedrich's Ataxia and Ubiquitin-Competing Molecules

Friedrich'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 in 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 that bind to FXN compete with ubiquitin for binding to FXN (at a specific site on the molecule, lysine 147) and lead to the 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 [56]. 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 [56]. Conroy et al. [56] suggested the hypothesis that interference with the 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 the gene *KCNA1*, which codes for a potassium channel protein [89]. KCNA1 has been recently identified as having E3 ubiquitin ligase activity [61] (see Supplemental Table 4 in this reference). AS noted above, EA subtype 2 is caused by a 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 have 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 (ASDs) [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 the dysregulation of protein degradation in ASD. The number of ubiquitin–proteasome proteins listed as risk factors may reflect the heterogeneity of 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 indicated whether they were significantly upregulated or downregulated in the various subgroups of the Thompson supplementary dataset. We also noted the 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 differential 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 MB subtypes. In Table 1, we list the ubiquitin conjugases that were significantly upregulated or downregulated in the Thompson dataset compared to the other MB groups. Thus, E2 conjugases, 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 of 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

Table 1 Ubiquitin-conjugating enzymes in MD groups of Thompson et al. [111]

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

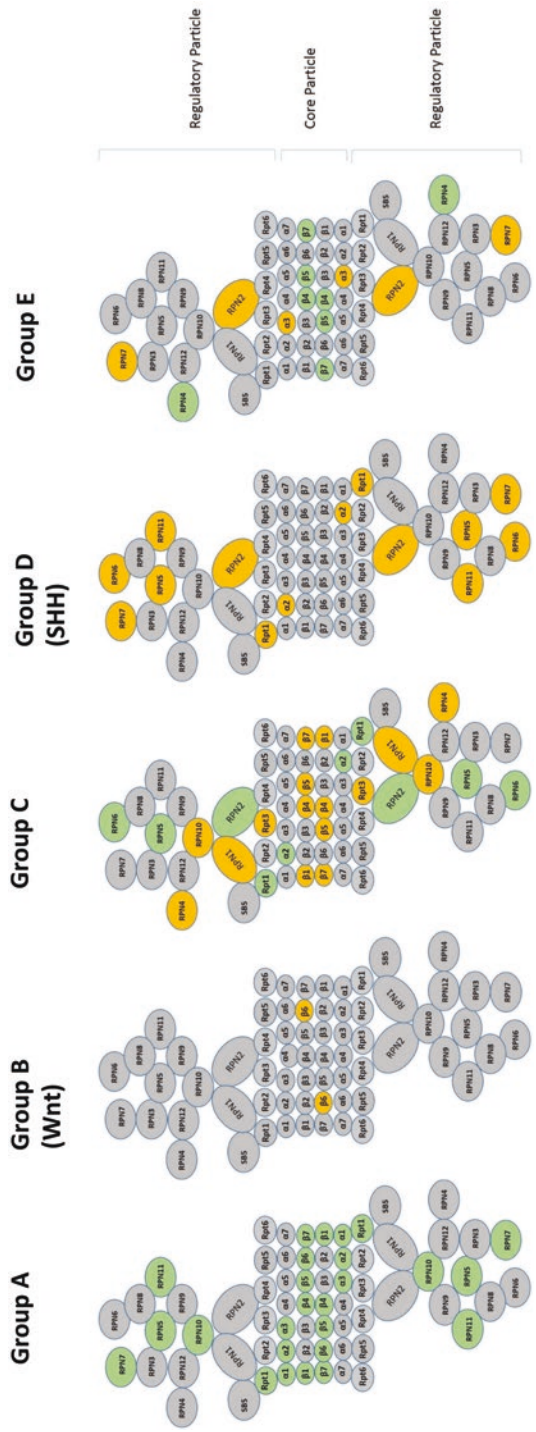

Fig. 1 Differential expression of proteasome subunits in MB subtypes in Thompson supplemental dataset. (Used with permission, Springer, Cellular and Molecular Life Sciences, 2016)
Green, significantly increased; orange, significantly decreased compared to other groups; gray, not in the dataset or not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

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.

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, 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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Epigenetic Control and Cerebellar Neurodevelopmental Disorders

Mojgan Rastegar

Abstract Epigenetic mechanisms regulate cellular identity and organ morphology *via* instructing 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, crosstalk between DNA and histone marks, nucleosome positioning, chromatin remodeling, enhancer–promoter interactions, as well as the 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 to the forefront of cutting-edge research for therapeutic strategies. One challenge would be the very large number of genes that will be targeted by most epigenetic drugs that are capable of global modulation of epigenetic marks. Thus, purposeful management of selectively targeting disease-associated genes in balance with the global effects of epigenetic drugs should be considered.

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

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Introduction

The 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 the "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 to chromatin as "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 >129,000 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 modifications (PTMs), 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 PTMs, while discussing the crosstalk of these two types of epigenetic modifications. I will then briefly overview other types of epigenetic mechanisms such as unidirectional chromatin remodeling and bivalent histone marks at the developmentally important *Hox* genes. Lastly, I will discuss some examples of neurological brain disorders with an epigenetic or epigenetic–genetic basis to provide some views on the future directions 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 cellular morphologies throughout life and dictates its "phenotype" [3]. The term "epigenetics" is primarily rooted from the Greek word "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 throughout embryonic development and after birth during infancy,

childhood, and adulthood, through life [4]. By regulating the gene expression program of individual cells, epigenetic information determines and dictates 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]. Deregulation of epigenetic mechanisms is involved in both common and rare human diseases [6–8]. 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 malnutrition or stress after birth with a negative impact on the body and the brain. Accordingly, environmental factors can manipulate and reorganize the composition of epigenetic marks on DNA molecules. This may lead to a different outcome in cellular gene expression program causing 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 involvement of environmental factors in human disease and neurological disorders.

DNA Methylation

Perhaps the very first evidence of epigenetic modifications goes back to 1963 when DNA and RNA methylation was primarily noticed [9]. However, the discovery of eukaryotic DNA methylation happened 14 years later and through the research of Razin and Cedar in 1977 [10]. Today, there are over 94,000 published original research and review articles on DNA methylation, capturing the impact of these discoveries over the last 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 crosstalk of "histone code" and DNA methylation, and lastly "gethylome/gethylomics" that connects "methylome/methylomics" to "genome/genomics" [11–13]. Research by independent groups have 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 [14]. As expected, deregulation of these epigenetic mechanisms and/or mutation in the components of epigenetic machinery are associated with human disease, cancer, and neurological disorders.

Chemically, DNA methylation is characterized by the covalent binding of a methyl group (CH3) to the 5th 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 referred to as the 5th base of genomic DNA and is commonly

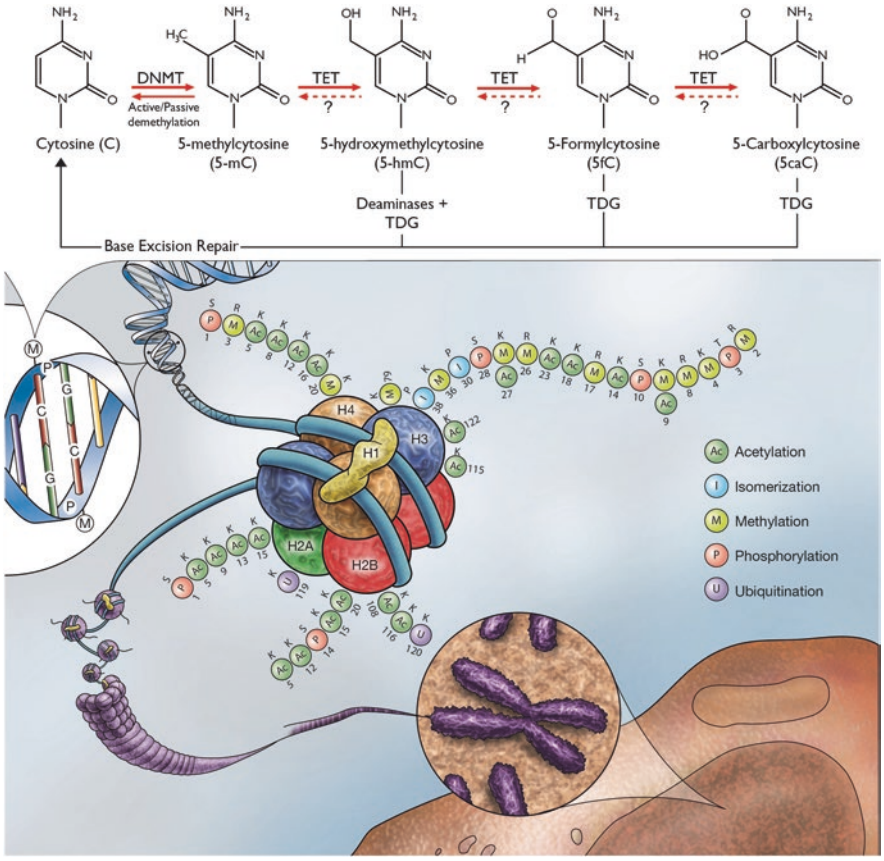

Fig. 1 The basic structure of a nucleosome, different types of DNA methylation, and diverse forms of histone post-translational modifications (PTMs) are shown. The histone octamer of 2X H2A-H2B, two molecules of histone H3, and two molecules of histone H4 are shown with the double-stranded DNA molecules and histone H1 that connects the adjacent nucleosomes. Different types of histone PTMs are shown, along with DNA methylation at the CpG dinucleotides. At the top, formation of different types of DNA methylation through the action of DNMT and TET proteins are also shown. (This figure is adapted and modified from Rastegar and Barber (2010) [65], and taken from the previous edition of this book chapter (Rastegar 2017) with the editor's permission [116])

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 [11, 15]. The CpH methylation is relatively abundant in the brain and in neurons, but still much below the rate that CpG methylation occurs; for example, in adult mice brain neurons, the ratio of CpG methylation is about ~75%, while CpH methylation is ~25% [16].

In 2009, independent research groups reported a new type of DNA methylation known as 5-hydroxymethyl cytosine (5-hmC) [17, 18], which is now referred to as

the 6th base of the genome [19, 20]. The newly identified 5-hmC is highly enriched in embryonic stem cells and Purkinje cells of the cerebellum [17, 18]. 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) [21]. 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), with some advancement in research about their functional role [22, 23].

DNA Methyl Writers

Members of the DNA methyltransferase (DNMT) family are responsible for the deposition of the methyl CH3 modification on cytosine nucleotides to form a 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 [24]. DNMT3A and DNMT3B establish the primary framework of CpG DNA methylation [25, 26], without favoring the hemi-methylated *versus* unmethylated DNA. The third member of this group is called DNMT3-like protein (DNMT3L) and is considered to be an enzymatically inactive member [26]. In mice, transgenic *Dnmt1*-deficiency causes widespread genomic DNA demethylation that leads to embryonic lethality rapidly after gastrulation and during early embryonic development [27]. 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 [28]. In contrast to these two members of the DNMT3 group, mice with *Dnmt3L*-deficiency survive until adulthood; despite the fact that male knockout mice are infertile as their sperms do not mature [29].

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-deafness and Narcolepsy (ADCA-DN)" [30, 31]. *DNMT3A* mutation is associated with overgrowth disorders [32], and *DNMT3B* mutations are connected to ICF (immunodeficiency, centromere instability, facial abnormalities) syndrome, which is a rare autosomal disease [33].

DNA Methyl Readers and MeCP2

Once 5-mC DNA methylation is established, this epigenetic modification will be recognized, bound, and interpreted by the family members of the methyl-binding proteins (MBPs). 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 Dr. Adrian Bird and his team in 1992 [34].

By binding to 5-mC, MeCP2 represses its downstream target genes *via* multiple mechanisms [35, 36]. The 5-mC commonly marks inactive genes, which are critically important for transcriptional silencing, imprinting, X-chromosome inactivation, genomic stability, embryonic development, and proper function of the brain [5, 37]. *In vivo* studies in the mouse brain show that MeCP2 specifically binds to 5-mC at the genes that carry DNA methylation [38]. *In vitro* DNA-protein binding assays show that MeCP2 preferentially binds to methylated DNA and has a low affinity for unmodified DNA. The high-affinity binding of MeCP2 to 5-mC requires MeCP2 N-terminal regions and its methyl-binding domain (MBD) [39]. MeCP2 DNA binding activities are essential for the proper chromatin structure formation in neurons, where the protein is exceptionally abundant [35]. 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 [38]. However, in the absence of MeCP2, a few critical target genes (such as *Bdnf*) are always and specifically altered in a cell type- and brainregion-specific manner [40–42], highlighting the role of MeCP2 as a target-specific transcriptional regulator.

Adding a new layer of complexity to the MeCP2 function is the discovery that MeCP2 is capable of binding to 5-hmC in the brain. As stated earlier, 5-hmC is an abundant DNA modification in the brain and is suggested to mark active genes [43]. This is in direct contrast to 5-mC, which 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 MBD mutations, MeCP2 R133C mutation only loses 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 [43]. In addition, as a transcriptional repressor, MeCP2 is also reported to act as an activator of transcription [44]. Accordingly, it is suggested that MeCP2 acts as a repressor when bound to 5-mC, and as an activator when bound to 5-hmC [43].

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. The X-linked *Mecp2/MECP2* gene produces two functional protein isoforms, called MeCP2E1 (also named MeCP2B or MeCP2α) and MeCP2E2 (also named MeCP2A or MeCP2β) with unique N-terminal sequences. These isoforms are generated through alternative splicing of the second exon [45]. In the brain, distinct transcript expression patterns are detected for individual isoforms [46], with *MECP2E1* displaying 10× higher expression levels [47]. The difference at the N-terminal sequences of MeCP2 isoforms is rather short, with 21 amino acids exclusive to MeCP2E1 encoded by

exon one, and 9 amino acids only present in MeCP2E2 encoded by exon two. The regulation and functional properties of the two MeCP2 isoforms have been the subject of my laboratory for over a decade. By generating isoform-specific antibodies, my team reported that MeCP2E1 is the major protein isoform in the murine brain, with significantly higher expression in primary cortical neurons than in astrocytes [48]. We reported that MeCP2E1 protein is uniformly expressed in different brain regions, while MeCP2E2 displayed a brain region-specific expression pattern. One brain region that showed the highest expression level of MeCP2E2 (almost similar to MeCP2E1 levels) was the cerebellum, highlighting the functional importance of both MeCP2 isoforms in this part of the brain [49]. We further reported that during differentiation of embryonic brain-derived neural stem cells, *Mecp2*/MeCP2 expression is controlled by DNA methylation, with a reciprocal expression pattern [50, 51]. Importantly, a significant correlation exists between the transcript and protein expression of the two MeCP2 isoforms with that of DNA methylation at its regulatory DNA sequences (regions one to six: R1-R6) in the adult murine brain [49]. I will further discuss the importance of MeCP2 and its two isoforms in the human disease and neurodevelopmental brain disorders in the last section of this book chapter.

DNA Methyl Erasers

Originally, DNA methylation was considered a stable and repressive type of epigenetic modification. However, it is now clear that through the action of Tet 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). Mice deficient for either of *Tet* genes (*Tet1*$^{-/-}$, *Tet2*$^{-/-}$, *Tet3*$^{-/-}$) have normal preimplantation development. While *Tet3*-deficiency leads to neonatal lethality after birth [52], *Tet2*-deficient mice develop spontaneous myeloid leukemia [53, 54], with *Tet1*-deficient mice only showing a small body size. Nevertheless, despite being a mild phenotype, the small body size of *Tet1*-deficient mice is noticeable from the postimplantation stage and during embryonic development [55].

Regarding the role of 5-hmC, research studies suggest that it promotes transcriptional activation due to high abundance in the intragenic regions of the enhancers and transcriptionally active genes in embryonic stem cells [21, 56]. During early development, passive DNA demethylation occurs through consequent cellular divisions in the absence of DNA methyl transferases (Fig. 2). This is associated with global DNA demethylation of 5-mC at the paternal genes, right after fertilization and in the early preimplantation embryos due to active and passive 5-mC DNA demethylation. Passive 5-mC demethylation coincides with the absence of DNMT1 transcription and expression, as well as dilution of the oocyte-originated DNMT1

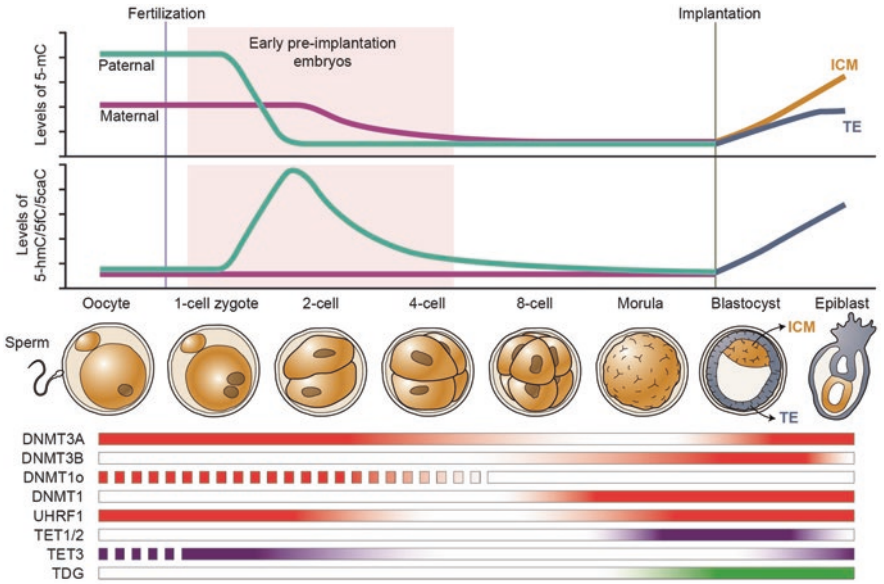

Fig. 2 Global change in different types of DNA methylation in the paternal and maternal genes during the preimplantation period of development in mouse embryos. The global change in the levels of different types of DNA methylation (5-mC, 5-hmC, 5fC, and 5caC) is shown. The relevant expression patterns of DNA methyl transferases (DNMT) 3A, DNMT3B, DNMT1, DNMT1^{o} (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). (The figure is modified and adapted from Wu and Zhang (2014) [57] and taken from the previous edition of this book chapter (Rastegar 2017) with the editor's permission [116])

(DNMT1^{o}). The level of DNMT1 during this time is diluted in every cell cycle and reduced as the cells divide. Passive DNA demethylation is due to DNMT1 absence, even though 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 the subsequent enzymatic activity of DNAM1. On the other hand and in parallel, there is an active 5-mC DNA demethylation at the paternal genes due to the activity of TET proteins in oxidizing 5-mC to 5-hmC, 5fC, and 5caC. TET activities during this embryonic developmental time 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 of development. After the formation of 5fC and 5caC, a destabilization of the N-glycosidic bond may happen that promotes thymine DNA glycosylase (TDG) activity *via* specific chain of events that lead to base excision repair (BER) and removal of the modified nucleotide, but such a 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 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 lacks DNA methylation until 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 happens with 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, 57].

Histone Modifications

Perhaps the most diverse form of epigenetic regulation is orchestrated through histone post-translational modifications. Histones are highly alkaline DNA-bound proteins that make up the octamer core of the nucleosomes (two dimers of [H2A-H2B] + two molecules of histone H3 + two molecules of histone 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 the addition of histone molecules would bring it to about 90 μm in the form of chromatin structure within the cellular nuclei [58]. The very first histone PTMs that were reported in 1964 consisted of the acetylation and methylation of histone molecules [59]. Today, we know of many other forms of histone PTMs beyond histone acetylation and histone methylation that include histone phosphorylation, isomerization, ubiquitination, and ADP-ribosylation (Fig. 1). Such histone modifications are usually at the terminal domain of histone molecules, except for a few histone PTMs that may also occur within the core part of the histone molecules (i.e., phosphorylation of histone H3 tyrosine 41). Considering a 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 that the addition of mono-, di-, or trihistone modifications, which may not necessarily mark the same chromatin compartments, and within the di-modifications, there are also potentially symmetric or asymmetric histone PTMs that once again may be a signature of different chromatin compartments. Together, with crosstalk of histone PTMs and DNA modifications (5-mC, 5-hmC, 5fC, 5caC, and CpH methylation), the potential physical masking of one histone PTM by another PTM, regulatory role of three-dimensional chromatin structure, chromatin remodeling, and nucleosome positioning; the magnitude and complexity of such orchestrated molecular mechanisms in every single cell of the body can be appreciated.

Like what was discussed for DNA methylation in the previous sections, there are "writers," "readers," and "erasers" of histone PTMs as well. Depending on the type

of histone PTMs, the proteins that are within each group of the “writers,” “readers,” and “erasers" are different. For examples, histone acetylation of lysine amino acids is catalyzed through 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 histone PTMs, which may subsequently recruit other transcription factors or regulatory molecules to communicate the intended epigenetic signal in transcriptional activation. Histone acetyl “eraser” molecules are grouped as members of the histone deacetylase (HDAC) family that belong to HDAC class I to IV. For detailed information about members of this family of erasers, please refer to other resources and their references [11, 60, 61]. 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 PTMs that are discussed in detail elsewhere [11, 24, 62].

Other Epigenetic Regulatory Mechanisms

Besides DNA methylation, histone modifications, and their crosstalk, 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, Piwi-interacting RNAs) in transcriptional silencing and translational regulation, long noncoding RNAs in transcriptional control, 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 [5, 63]. Misregulation of these molecular events may lead to impaired cellular function, cancer, and human disease. Perhaps the best-studied examples of developmentally important genes that are controlled by such an orchestrated cascade of epigenetic mechanisms take place at 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 a combination of DNA methylation marks, histone PTMs, a suggested unidirectional chromatin remodeling (shown experimentally for *Hoxd4*) [64] (Fig. 3b), enhancer–promoter integrations, nucleosome positioning, and the activity of regulatory RNA molecules. For a detailed review of the *Hox/HOX* gene control by epigenetic mechanisms, refer to other resources and their references [65–67].

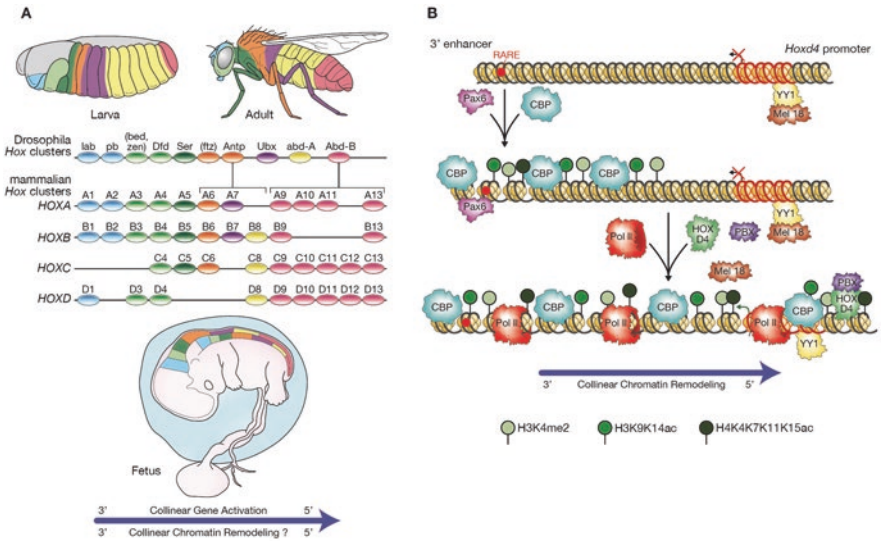

Fig. 3 Schematic illustration of the *Hox/HOX* clusters and unidirectional chromatin remodeling at the *Hoxd4* gene locus. (**a**) The larvae and adult *Drosophila Melanogaster* are shown on the top. The color-coded fly is in harmony with the *Hox/HOX* gene members of the 13 paralogue groups shown below the fly images. Members of each paralog group (i.e., *Dfd*, *HOXA4*, *HOXB4*, *HOXC4*, and *HOXD4*) are more similar to each other in comparison to the members from the same *HOX* cluster (i.e., *HOXA1* to *HOXA13*). Underneath the fetus, a proposed unidirectional chromatin opening at the 3′ to 5′ unidirectional *HOX* gene activation (collinearity 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 the *Hoxd4* promoter located more at 5′ to its enhancer is shown. Note that the recruitment of the CBP (histone acetyl transferase) and the transcription factor PAX6 and the initiation of H3K9ac, H3K4me, followed by H4K4K11K15ac 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 [64, 71, 72]. (**a** and **b** are modified and updated from Barber and Rastegar (2010) [65] and taken from the previous edition of this book chapter (Rastegar 2017) with the editor's permission [116])

Epigenetics in Development and Bivalent Marks

Epigenetics plays 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 efforts from independent research groups have been devoted to understanding the biology of global DNA demethylation at both maternal and paternal genes, which happens because 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 active DNMT1

transcription and translation of new molecules, along with dilution of oocyte-originated DNMT1^{o} during each mitosis cycle. 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 (5-hmC/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 inner cell mass (the cellular mass that in the primordial embryo 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 different DNA methylation types and other epigenetic mechanisms tightly control the proper process of embryonic development. Deregulation of these mechanisms due to genetic mutations or influenced by environmental factors may lead to mild-to-severe consequences in the developing embryos.

To understand early developmental mechanisms 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 PTMs (Fig. 4). It has been shown that *Hox* genes and some developmentally important genes are at a poised state of transcription, carrying "bivalent marks" within the "bivalent domain" 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 [68]. The "bivalent marks" are accordingly referred to the existence of these active (H3K4 methylation) and inactive (H3K27 methylation) marks at the N-terminal region of the same histone H3 molecule at the regulatory regions of developmentally important genes [65]. 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, 64, 65, 69–72]. As development/embryonic stem cell differentiation proceeds, bivalent marks at the poised genes are resolved to further gain DNA methylation in combination with keeping the H3K27 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) transcriptional silencing proteins. The pluripotency genes further control their own expression through a regulatory feedback loop during embryonic stem cell self-renewal. For a detailed review of the bivalent marks, please refer to the following reviews/book chapters and the references in there [5, 24, 73]. Depending on the process of differentiation toward

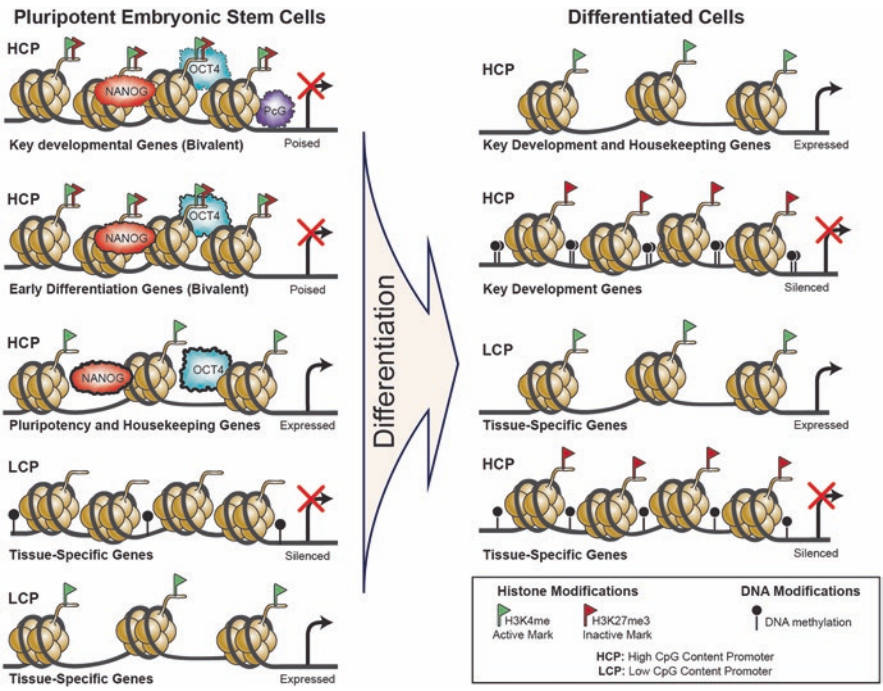

Fig. 4 Presence of bivalent marks in the embryonic stem cells and their change of status in differentiated cells. Bivalent marks (active: H3K4me3, and inactive: H3K27me3) exist on the N-terminal tail of the same molecule of histone H3 in the pluripotent embryonic stem cells at the high CpG content promoters of the developmentally important genes (i.e., *Hox* genes) and early differentiation genes. The thickness of the black line for NANOG and OCT4 correspond to the enrichment of these transcription factors and/or 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. (The figure is modified and updated from Delcuve et al. (2009) [116] and taken from the previous edition of this book chapter (Rastegar 2017) with the editor's permission [116])

different cell fate commitments, epigenetic marks and transcription factor binding at the *cis*-regulatory elements would change to ensure the proper gene expression program of individual cells. Once the central nervous system and the brain development process are completed, bivalent marks barely exist at the *Hox/HOX* genes; for example, the only remaining *Hox* gene with a bivalent modification is *Hoxa1* (Fig. 5).

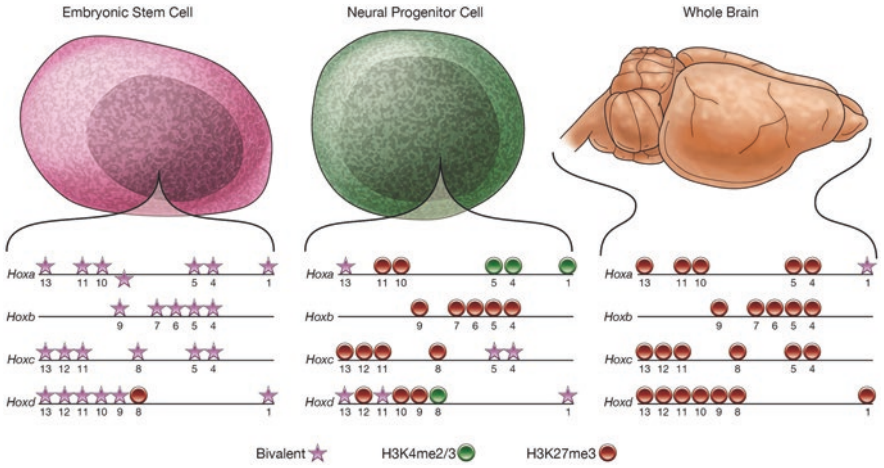

Fig. 5 Bivalent marks at the *Hox* clusters during the development of the brain cells. In pluripotent embryonic stem cells, bivalent marks exist at all paralogue members of the *Hox* genes along all four clusters, except for the *Hoxd8*. Later during the differentiation of the brain cells in neural progenitor 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). (The figure is modified and updated from Barber and Rastegar (2010) [65] and taken from the previous edition of this book chapter (Rastegar 2017) with the editor's permission [116])

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 the brain in the posterior cranial fossa. Anatomically, 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 the 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 reactions 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 [74]. Among different modes of epigenetic mechanisms, DNA methylation has attracted much attention in cerebellum development and function [75]. Within 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 [43]. In the adult mouse brain, reports from my own lab indicated that both MeCP2E1 and MeCP2E2 are expressed in neurons,

astrocytes, and oligodendrocytes. However, expression of the minor MeCP2 isoform (MeCP2E2) reaches its highest level in the cerebellum with almost comparable levels to the major MeCP2E1 isoform [49]. Of note, a Daoy medulloblastoma cell line originating from the cerebellum has been useful in studying *MECP2*/MeCP2 regulation and function [76–78]. Daoy cells represent the sonic hedgehog (SHH) medulloblastoma subgroups [79, 80].

Epigenetics and Neurodevelopmental Cerebellar Disorders

Deregulation of epigenetic mechanisms in the brain and cerebellum are associated with neurodevelopmental and cerebellar disorders. Research by independent groups has established the involvement of epigenetic mechanisms in FASD, autism spectrum disorders (ASD), fragile X syndrome, and Rett Syndrome (RTT). Of the 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 (see chapters "Teratogenic Influences on Cerebellar Development" and "Neurodevelopmental Disorders of the Cerebellum: Autism Spectrum Disorder").

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 influences 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 the cerebellar vermis of developing mice are 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 a significant decrease in the number of cells, dendritic arborizations, and/or axonal projections [81]. 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 [50, 82–85]. The associated genetics and epigenetics of FASD are discussed in detail elsewhere [86] and impaired cerebral function in FASD.

Studies in human postmortem 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 [87]. Such epigenetic change and altered MeCP2 binding were not detected at the *GAD1* and *RELN* gene bodies of ASD patients in the same examined cerebellum samples. While the protein product of the *GAD1* gene is involved in gamma-aminobutyric acid (GABA) synthesis, REELIN is dominantly expressed in glutamatergic neurons [88]. In postmortem brain tissues of ASD patients, GABA neurotransmitters are at reduced levels in the cerebellum [89, 90]. Research by independent groups has suggested the role of DNA methylation in ASD and the potential application of DNA methyl inhibitors for ASD [51, 91, 92].

In one study of human suicides cases 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 was not detected in the cerebellum. The authors concluded that extensive cerebral astrocyte dysfunction is associated with major depressive disorders [93]. Other studies further introduced DNA methylation as an emerging marker for cerebellar epigenetic age [94]. 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 links 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 role of Purkinje cells in this regard, and specifically on their vulnerability to *ATM*-deficiency. However, research 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 the loss of 5-hmC is critically important in mediating the susceptibility of Purkinje cells to *ATM*-deficiency [95].

Within neurodevelopmental and cerebral disorders with an epigenetic link, perhaps one of the most-studied diseases would be Rett Syndrome. 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 [96]. 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 ASD [36, 97–101]. Aberrant MeCP2 expression in the brain also leads to compromised brain function and ASD [102]. Currently, RTT 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 [103, 104]. 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 [105]. To date, there have been many attempts to find therapeutic strategies for RTT 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 an RTT mouse model [106]. These studies have established that despite MeCP2 functional role in the epigenetic silencing of gene therapy vectors in stem cells [107], 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 [106]. This was one of the first studies to support the rescue role of MeCP2E1 in

correcting the impaired morphology of *Mecp2*-deficient neurons [106]. Subsequent studies from independent groups indicated that both MeCP2E1 and MeCP2E2 can rescue RTT-associated phenotypes in mice but with different efficiencies [108]. 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 influenced by DNA methylation, involving both 5-mC and 5-hmC [49–51, 85, 109]. MeCP2 is an interesting protein that links genetic mutations and epigenetic regulation to cell signaling molecules. Indeed, *MECP2* loss-of-function mutations lead to impaired mTOR pathway in postmortem human cerebellum [110]. As the *Mecp2/MECP2* gene is X-linked, it is not surprising that its expression level is sex-dependent [109, 111] and that MeCP2-associated disorders are differently detected in males and females. In this regard, RTT is primarily a female disorder, whereas *MECP2* duplication syndrome (MDS) is detected in males [36, 112, 113]. Proper regulation of *MECP2*/MeCP2 isoforms is important in the brain, and RTT patients exhibit deregulation of *MECP2E1-E2* homeostasis regulation [114, 115]. 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 MeCP2 isoforms in RTT and other neurodevelopmental cerebral disorders.

Conclusions

Epigenetics controls and dictates the identity of individual cells during each cellular division. Throughout development, cellular programming 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 for today's research.

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Hormonal Regulation of Cerebellar Development and Its Disorders

Noriyuki Koibuchi

Abstract Cerebellar development and plasticity is involved 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 patterns of intrinsic gene expression tightly regulated by their molecular programs. The latter includes sensory inputs, mediated by the peripheral nervous system and

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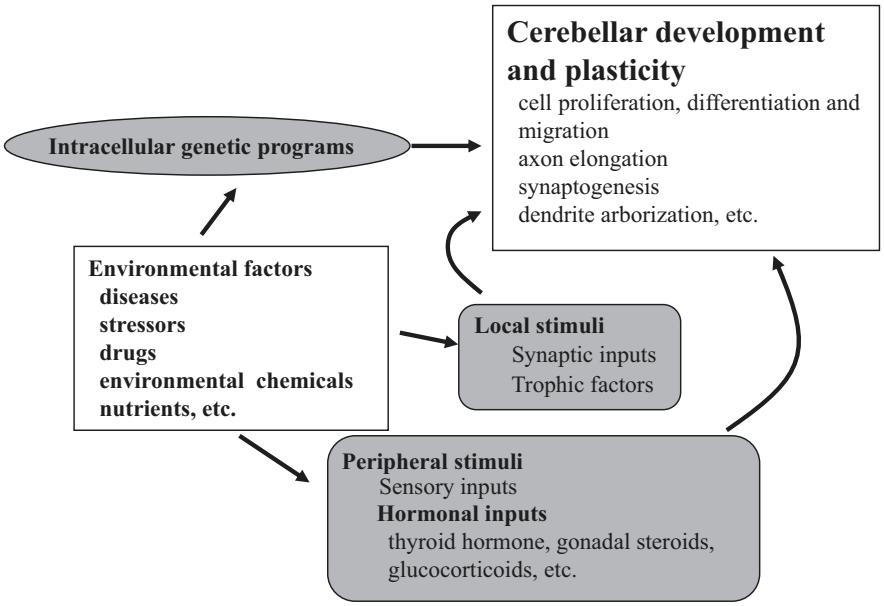

Fig. 1 Schematic diagram showing genetic and epigenetic influences and its modulation by environmental factors involved in cerebellar development and plasticity

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.

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 the 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 the dimerization of NRs and ligand-dependent transactivation (activation function-2, AF-2). To activate or repress the transcription of the 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 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, the involvement of TH (triiodothyronine [T_3] and thyroxine [T_4]) on cerebellar development has been well studied. Deficiency of TH during 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

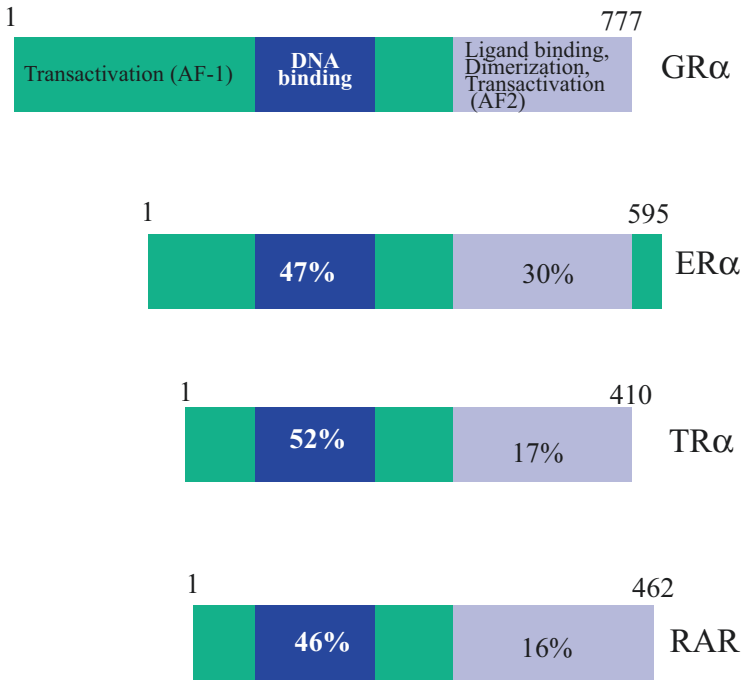

Fig. 2 Protein sequence homology among representative nuclear hormone receptors. *ERα* estrogen receptor alpha, *GRα* glucocorticoid receptor alpha, *RAR* retinoic acid receptor, *TRα* thyroid hormone receptor alpha

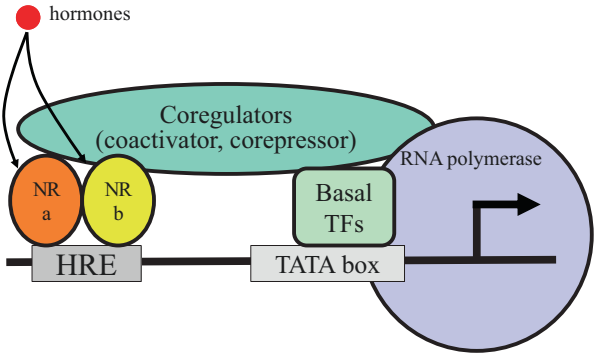

Fig. 3 Interactions of nuclear receptor (NR), transcriptional coregulatorsand basal transcriptional machinery such as basal transcription factors (TFs). NRs bind to hormone response element (HRE) located in the promoter region of target genes

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 glucocorticoids, 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 periods 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 1/3000–1/4000 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].

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 NT-4/5, 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 knock-out mice, TRβ knock-out mice, and TRα/TRβ double knock-out 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 an 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, which transports T_3 into the neurons [31]. Animal studies have shown the disruption of

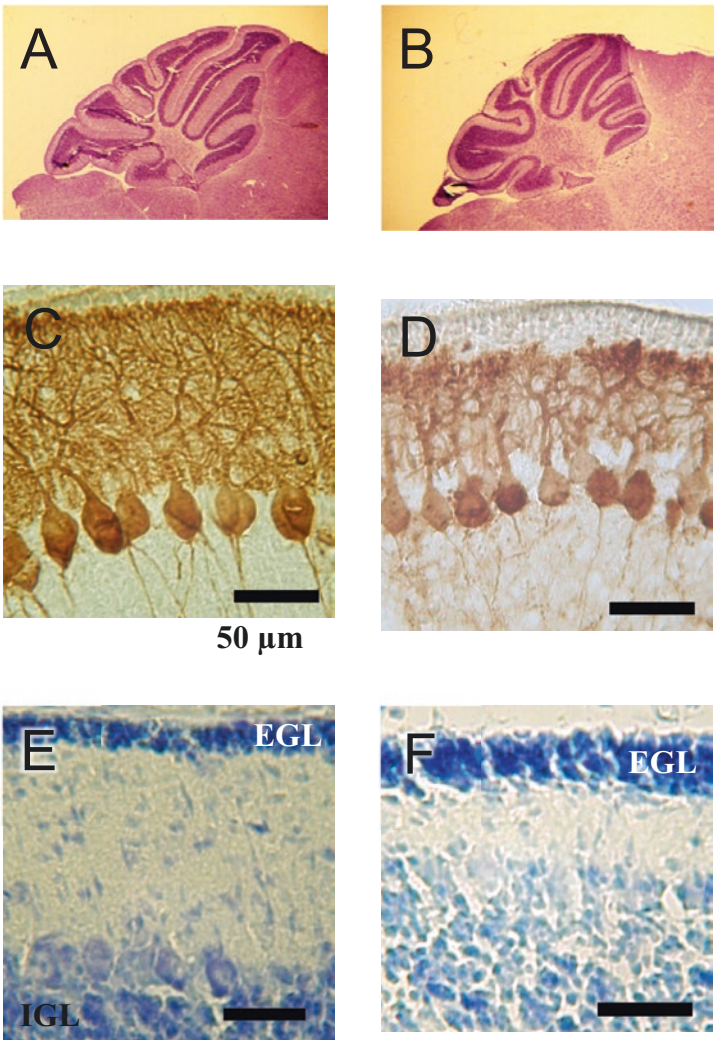

Fig. 4 Effect of congenital hypothyroidism in rat model. Rdw congenital hypothyroid rat, which harbors mutated thyroglobulin gene, shows delayed cerebellar development (**b**, **d**, **f**) compared to control animal (**a**, **c**, **e**). Note the decrease in dendrite arborization of Purkinje cell (**d**),and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

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 the 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 than in 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 the involvement of E2. However, studies of the aromatase gene using knock-out 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 in 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 a major gonadal steroid that may have some effect on the developing cerebellum. Previous studies showing the expression of aromatase in mid-gestation in monkeys [42] and early postnatal age in rats [43] support this hypothesis. Then, at 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 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; 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 a few examples related to sex 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

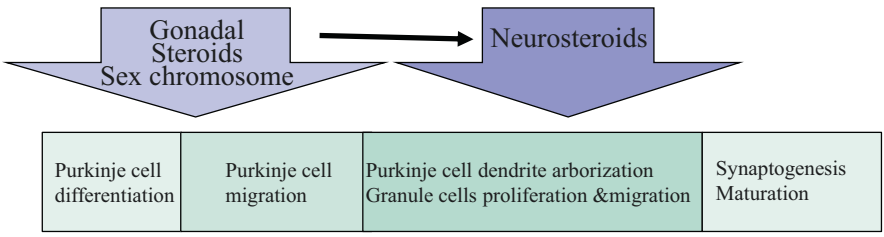

Fig. 5 Possible differential roles in gonadal steroids and neurosteroids during cerebellar development

hypoplasia in adults [52, 53]. In post-mortem 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 a 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 differences are unknown [59, 60]. To clarify the molecular mechanisms of sexual differences in cerebellar pathology, further study is necessary.

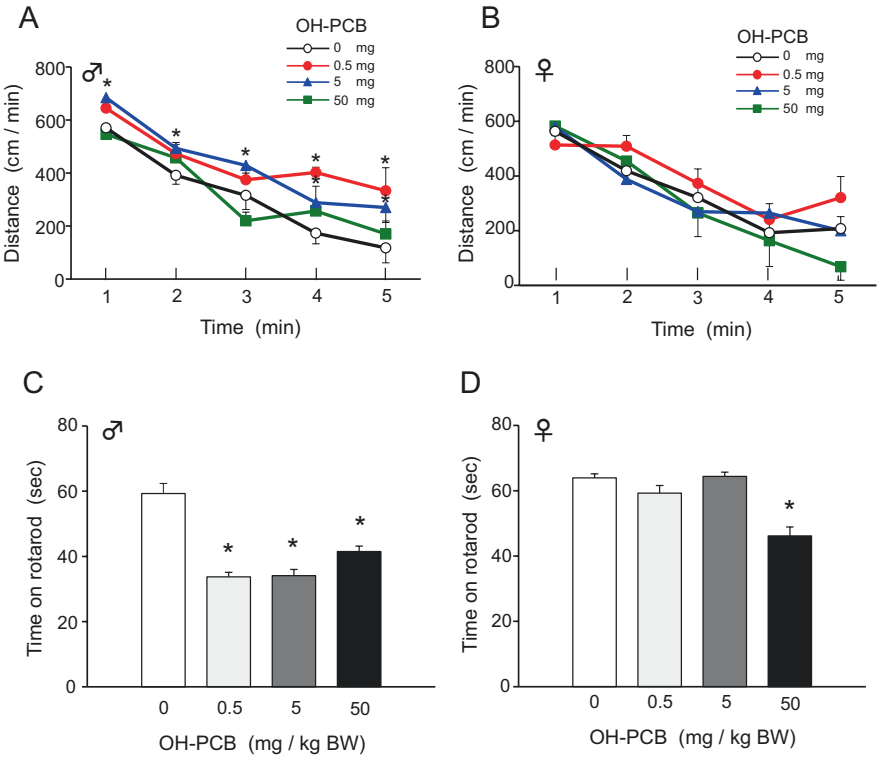

Fig. 6 Sexual difference in the effect of perinatal exposure to hydroxylated polychlorinated biphenyl (OH-PCB106). PCB was orally administered to the dam every other day from postpartum day 3 to 13 [58]. (**a**, **b**) Effects of PCB on locomotor activity in the open field in male (**a**) and female (**b**) rat. (**c**, **d**) Effect of PCB on motor coordination on rotarodin 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 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 nongenomic 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 cells becomes 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 and 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 develop alterations if glucocorticoid homeostasis is disrupted by perinatal stress or glucocorticoid administration. In rats, cortisone treatment during 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 receive 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 experiences, and the involvement of the 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 rats, 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 the high glucocorticoid 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 a such hormonal environment may affect such development. A large number of synthetic or natural chemicals may disrupt the 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 effects 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 1000 EDCs, more than 100,000 chemicals exist in the environment. The 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.

Table 1 Environmental chemicals showing hormonal or anti-hormonal activities

Classification	Chemicals
Pharmaceuticals	hormones or anti-hormones, 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 biproducts	Bisphenol-A, Polycarbonates, Butylhydroxyanisole(BHA), Chloro-& 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)

It should be noted that, because concentrations of 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 have been 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 through 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.

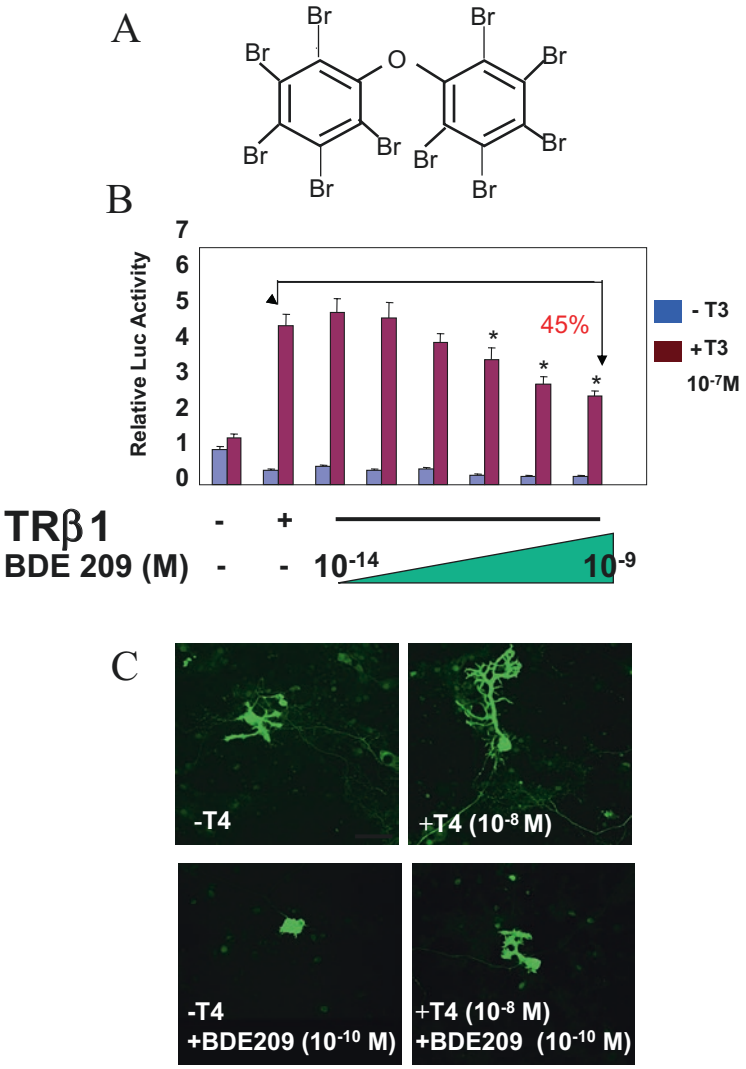

Fig. 7 Representative effect of EDCs (PBDE) on TH-mediated transcription and cerebellar development. (**a**) Chemical structure of BDE209. (**b**) BDE209 suppressed TRβ-mediated transcription, studied by reporter gene assay. (**c**) Effect PBDE (BDE209) on TH-induced Purkinje cell development in primary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

Conclusion

Although many hormone receptors are expressed in the developing cerebellum, only a limited amount of data is available in this regard. This may be a result of the challenges related to the 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 cascades may be involved to express their action as a specific phenotype. However, hormonal signaling plays an important role in mediating 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 (CNS), with more focus on the cerebellum.

Keywords Virus · Replication · Cerebellar ataxia

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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 (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 nm × 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 [32]. Several viruses, colloquially known as "giant viruses" (e.g., Mimiviruses and Pandoraviruses) can reach up to 1.5 μm in size [59].

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 *Coronaviridae* (which includes the SARS-CoV-2 virus responsible for the COVID-19 pandemic), *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 influenza viruses, members of the *Orthomyxoviridae* family, and the *Reoviridae*, 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 the *Nanoviridae* to successfully replicate, a cell must be infected with multiple particles that collectively provide all the genome segments [88].

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* 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

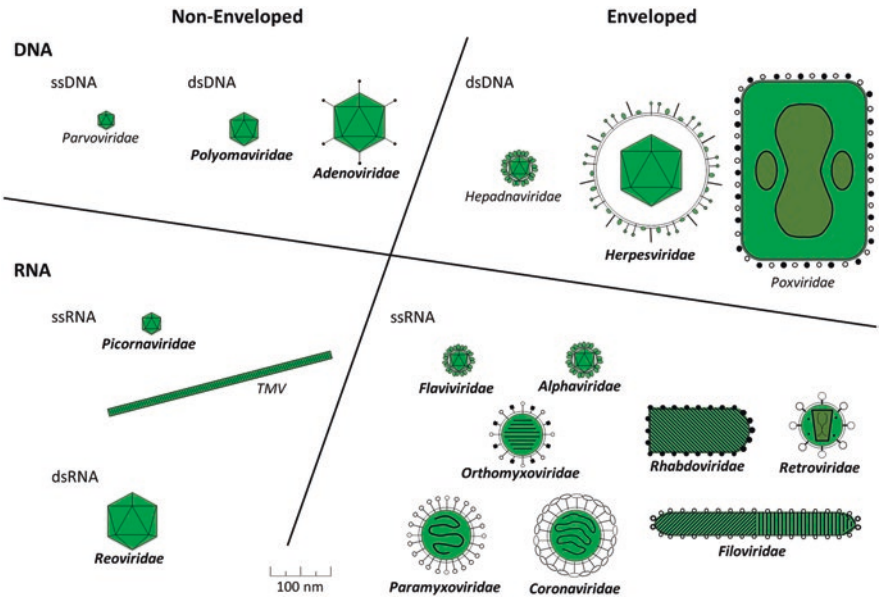

Fig. 1 Diagrammatic representations of selected virions. Viruses are divided according to whether their genomic material is DNA (top) or RNA (bottom) and whether the capsid is nonenveloped (left) or surrounded by an envelope (right). Where applicable, each group is further subdivided depending upon whether 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

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 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, messenger RNA (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 "Herpesviruses" and "Myxoviruses".

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 5000 nucleotides (= 5 kilobases, or 5 kb) and contain two genes. The largest human viruses (e.g., *Poxviridae* and *Herpesviridae*) can have genomes of 200 kilobase pairs 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 *non-structural*. 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, non-structural 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., [44, 80]), herpesviruses [60], filoviruses [90], and influenza viruses [85] also incorporate functional host-derived proteins into the virion. These host proteins may play important roles in the virus lifecycle [4, 27, 93].

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 semi-spherical 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), the *Coronaviridae* (e.g., SARS-CoV-2), 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 >3500 known virus species organized into >50 families [53]. 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 [7] 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 *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, SARS-CoV-2 in the *Coronaviridae* 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 enter the cell as part of the infecting virus. Class VI viruses (e.g., the retrovirus HIV) contain (+) ssRNA genomes that initially need to be converted into dsDNA by a viral-encoded reverse transcriptase. Class VII viruses (e.g., *Hepadnaviridae*) have a partial dsDNA

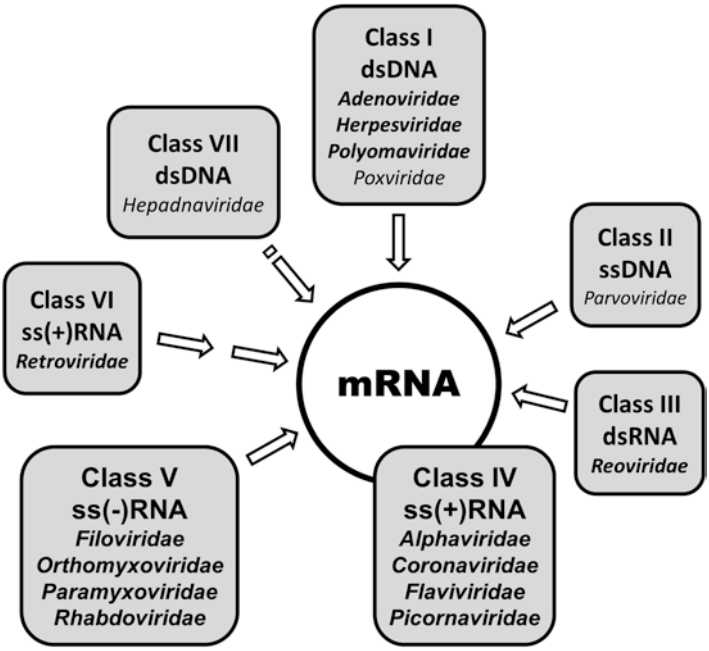

Fig. 2 The Baltimore transcription scheme classifies viruses based on their genomic nucleic acid content and on the strategies used to produce messenger RNA (mRNA). Virus genomes can be composed of RNA or DNA that is either single-stranded (ss) or double-stranded (ds). For RNA viruses, the polarity of the ssRNA can be either positive (+) or negative (−). By definition, mRNA is positive-stranded. Note that Class IV viruses have (+) RNA that can serve directly as mRNA as soon as the viral genome enters the cell. Class VI and VII viruses can undergo reverse transcription (RT) whereby RNA is converted into DNA. In the case of Class VI retroviruses, this happens via a DNA/RNA hybrid to generate the dsDNA intermediate (depicted as two arrows). In the case of Class VII hepadnaviruses (hepatitis B virus), the partial dsDNA must first be repaired to a complete dsDNA genome prior to mRNA synthesis (depicted as broken arrow). (Compiled and modified from http://viralzone.expasy.org/all_by_species/254.html)

genome that first is repaired into a complete dsDNA genome, transcribes various mRNAs, and 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 template to regenerate progeny dsRNA. Class IV viruses with (+) ssRNA genomes produce a full-length (−) ssRNA intermediate that is used as a 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 the 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 systems 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.

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 (Figs. 3 and 4). (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

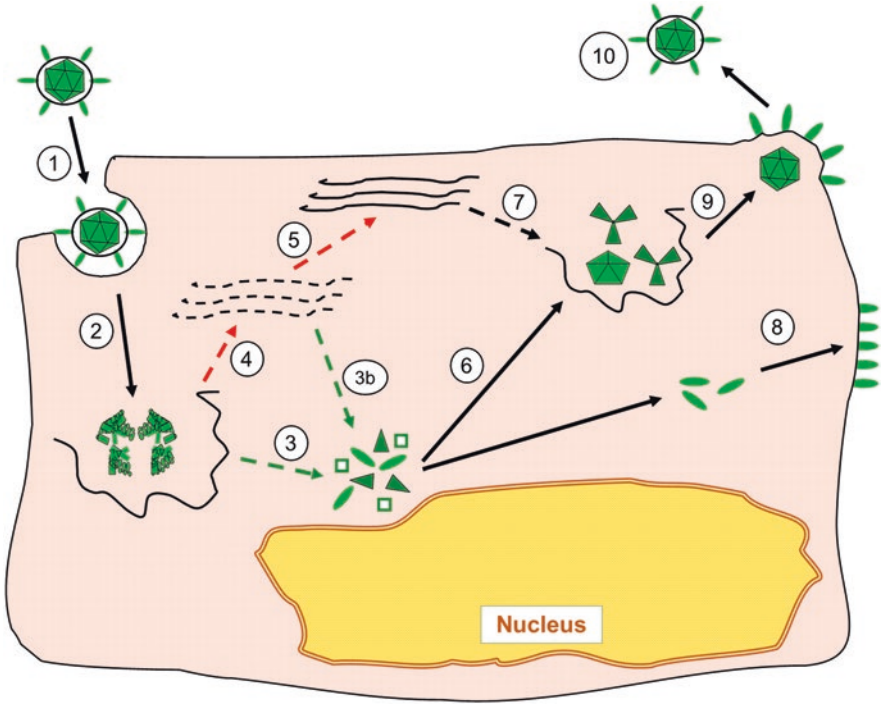

Fig. 3 Schematic representation of a typical viral replicative cycle for an enveloped RNA virus that acquires its membrane as it matures through the plasma membrane. Various steps are: *1*, Attachment; *2*, penetration into the cell, uncoating and release of genomic material; *3*, translation of mRNA and production of viral proteins; *4*, replication of genomic material to produce an intermediate of opposite polarity (dashed wavy line); *5*, replication of the intermediate to produce more genomes with same polarity as incoming RNA; *6*, association of viral proteins with *7*, replicated progeny genome; *8*, maturation of envelope proteins through Golgi and insertion into plasma membrane; *9*, assembly of progeny structural proteins and genomes into nucleocapsids which associate with regions of membrane containing viral envelope proteins; and *10*, maturation and virion release. If the incoming viral RNA is of (−) polarity, then the viral (−) genome is used to make mRNA (Step 4) before viral protein translation (Step 3b). Note that the locations of the various steps will vary depending on the virus type (e.g., DNA viruses usually carry out transcription and replication within the nucleus; see Fig. 4). Envelope acquisition for enveloped viruses can occur at intracellular membranes, or at the plasma membrane, as shown, upon release. Wavy lines represent viral nucleic acid, red arrows represent viral RNA processing, green arrows represent protein translation, green triangles and ovals represent viral structural proteins that assemble into complexes, small open squares represent viral non-structural proteins that are present within the cell and assist in viral replication and assembly but are not found within mature virions

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

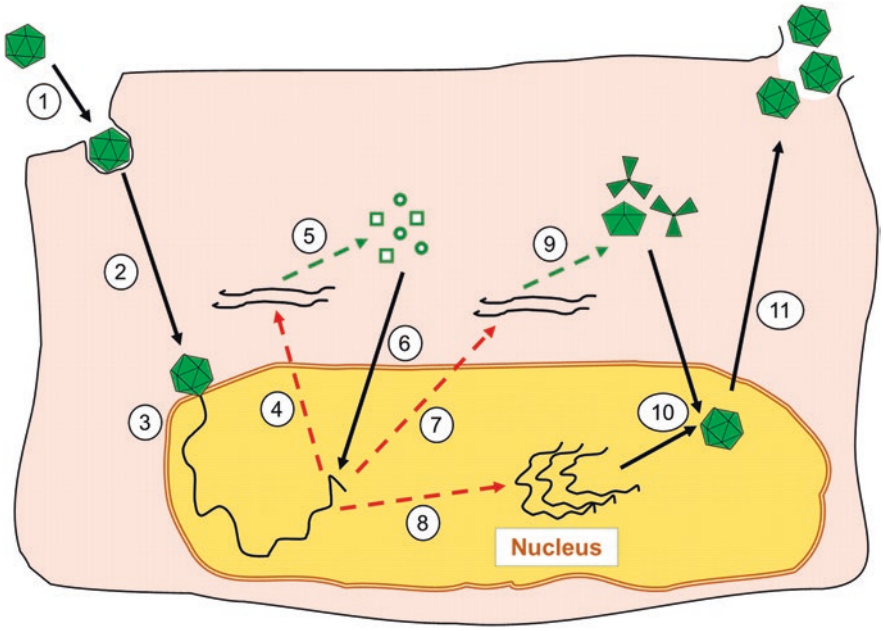

Fig. 4 Schematic representation of a typical viral replicative cycle for a non-enveloped DNA virus that performs many of its transcriptional and replicative steps within the cell's nucleus. Various steps are: *1*, Attachment; *2*, penetration into the cell and migration of viral capsid to the nucleus; *3*, injection of viral DNA into the nucleus; *4*, transcription of early mRNA which leaves the nucleus to be *5*, translated into early viral proteins which *6*, return to the nucleus where they promote *7*, transcription of late mRNA and *8*, replication of the viral DNA genome; *9*, translation of late viral proteins which return to the nucleus to *10*, associate with replicated progeny genome; and *11*, final maturation and virion release. Wavy lines represent viral nucleic acid, red arrows represent viral DNA and RNA processing, green arrows represent protein translation, green triangles represent viral structural proteins that assemble into complexes, small open circles and squares represent viral non-structural proteins that are present within the cell and assist in viral replication and assembly but are not found within mature virions

used (6) to make multiple (+) sense genomes. The progeny genomes aggregate with newly made viral capsid proteins (7) whereas viral envelope proteins (if the virus is enveloped) are shuttled through the cellular endoplasmic reticulum and Golgi apparatus (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 [57]. 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 CNS. One of the best known viruses, and that 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 [63], rabies primarily replicates in dorsal root ganglia, motor neurons, and sensory neurons in the spinal cord [26]. Viral antigens have been detected within the cerebellum shortly after infection [63] 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 [20]. 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 [65] 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 [72].

Arboviruses

A large number of different arboviruses (arthropod-borne viruses) are associated with neurological infections that include the cerebellum (reviewed in [81]). Arthropod-borne agents fall into several taxonomic groups and among viruses, arboviruses exist within at least 5 families [46]. The principle families are Alphatogaviruses (various equine encephalitis viruses including Eastern, Western, and 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 [58]. 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 [68]. Chikungunya virus has also been associated with cerebellar ataxia [49].

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 co-infection [50]. In addition, outbreaks of dengue fever in Sri Lanka involving tens of thousands of cases presented with acute cerebellar syndrome [104, 105]. Magnetic resonance imaging of eight encephalitic cases in India showed cerebellar involvement in all eight [41]. West Nile virus infection is also associated with neurological abnormalities in multiple cerebral compartments including the cerebellum [23]. Deer tick virus caused an extensive necrotizing meningoencephalitis that involved numerous brain regions including the cerebellum in a fatal encephalitis case [98]. The newly re-emerging Zika virus has been strongly associated with microcephaly and these brain malformations also include the cerebellum [24, 67, 78, 94, 101].

Coronaviruses

The world is currently experiencing one of its worst pandemics during the past 100 years. A novel coronavirus, named SARS-CoV-2 arose in Wuhan, China in late 2019 and has spread worldwide, causing coronavirus infectious disease 2019 (COVID-19).

The coronaviruses are a group of ssRNA viruses possessing unusually large genomes, of approximately 30 kb, and encoding an unusually large number of nonstructural genes [34] along with their four structural proteins. The four structural proteins are spike (S), which are large membrane proteins protruding extensively from the virion surface and appearing like solar "corona," hence the virus name, nucleocapsid (N), envelope (E), and membrane (M) [82]. The *Coronaviridae* family currently contains about 50 members, many of which infect livestock. There are at least seven human coronaviruses (hCoV). Several, such as strains 229E, OC-43, HKU1, and NL63 cause only mild cold-like symptoms in most infected individuals. A more pathogenic strain, named SARS (severe acute respiratory syndrome) appeared in 2003 and is estimated to have infected about 8000 individuals, leading

to 770 deaths (case fatality rate = 9.6%). Another pathogenic coronavirus, MERS-CoV (Middle East respiratory syndrome coronavirus) appeared in 2012, is estimated to have infected ~2500 individuals, leading to 860 deaths (case fatality rate = 34%). The latest pathogenic hCoV, SARS-CoV-2, has, to date, infected more than 250M individuals and led to more than 5.3M deaths [43].

The hCoV are generally considered respiratory viruses and predominantly spread that way. However, SARS-CoV-2 also affects a large number of organ systems and is associated with a large number of long-term sequelae [42, 103]. This is unusual for a "typical" ssRNA virus. Indeed, as indicated earlier, the hCoV are unusual in a number of ways. They encode an unusually large number of non-structural proteins, and while the functions of many are not yet fully known, it is likely that one or more are immunomodulatory, which could explain not only some of the long-term sequelae, but also explain why antibody responses to some SARS-CoV-2 proteins seem to wane relatively quickly [61, 62, 106].

Some of the viral-induced sequelae involve severe neurological and CNS manifestations [89]. These include anosmia, ataxia, confusion, dizziness, epilepsy, headache, hypogeusia, nausea, neuralgia, seizure, and vomiting [89]. Several case reports have found association between SARS-CoV-2 infection and cerebellar abnormalities [2, 18, 31]. For example, Guedj and colleagues examined two clinical cases and analyzed whole-brain ^{18}F-FDG PET; they reported an association between SARS-CoV-2 infection and hypometabolism in several brain regions, including the cerebellum [39] in one of the patients. Al-Dalamah et al. reported that a patient with relatively mild SARS-CoV-2 infection died from acute cerebellar hemorrhage [2] and Kirshenbaum and colleagues found cerebellar hemorrhages in four of six SARS-CoV-2-infected patients [54]. Ciolac and colleagues recently examined a young male patient and detected evidence of acute necrotizing encephalopathy by MRI in numerous brain regions including the cerebellum [18]. The patient recovered but presented several behavioral and cognitive perturbations.

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, 16, 83]. 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 [11, 36, 70, 77, 79, 86].

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 [25].

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 [97].

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-Jacob disease, and bovine spongiform encephalopathy-induced variant Creutzfeldt-Jacob disease. Abnormal prion protein deposition has been observed in Creutzfeldt-Jacob disease patients [33], other patients presented with cerebral cortical hyperintensity [47], and a recent quantitative proteomic screen identified substantial dysregulation of s-nitrosylated proteins within the cerebellum of prion-induced Creutzfeldt-Jacob disease patients [13].

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*) [52, 56, 99], JC virus (family *Polyomaviridae*) [22, 35, 96], parvovirus B19 [37, 38, 87, 100], and possibly adenovirus [74, 95]. Mild cerebellar signs were also seen in an Ebola virus-infected patient [15].

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 [12, 17, 76, 84]. 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 [77].

Mycoplasma pneumoniae has also been implicated in cerebellar ataxia [10, 40, 73]. 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 8 cases of cerebellitis [3]. *S. typhi* infection also leads to T1-weighted MRI hypointense cerebellar regions [71]. Infection and resulting cerebellar complications continue to occur, although antibiotic treatment of a few cases led to complete recovery [45].

Borrelia burgdorferi, the causative agent of Lyme disease, has also been associated with cerebellar ataxia, and with abnormal MRI lesions [5] and PET images [48] (reviewed in [30]). The bacterium *Tropheryma whipplei*, which causes Whipple's disease, has also been found to cause abnormal cerebellar MRI and ataxia [19, 64, 66].

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) [8, 51, 55, 69]. A recent report also identified cerebellar ataxia after vaccination with meningococcal group C agents [21].

Fungi

Several fungal species, including *Aspergillus* [14, 28, 29], *Histoplasmosis* [91, 102], *Candida* [92], *Exserohilum rostratum* [9], and *Phialemonium* [6], have also been associated with cerebellar infection [75, 77].

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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Interrelation Between the Immune and the Nervous Systems in the Context of Cerebellar Development and Developmental Disorders

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Abstract In recent years, the interplay between the development and function of the central nervous system and the immune system in the homeostatic and pathological state has become evident. Thus, understanding the crosstalk between the immune system and cerebellar development and functions has noticeable implications for managing neurodevelopmental, neurodegenerative, and neuroinflammatory disorders. In this chapter, we highlight the current progress of knowledge in the field of neuroimmunology and psychoneuroimmunology. Specifically, we discuss the contribution of the various immune responses in cerebellar development and its associated pathologies and highlight the current understanding of mechanisms involved in these processes. Immune pathways that play a crucial role in cerebellar development and functions are likely to become therapeutic targets for several neurodevelopmental, neurodegenerative, and neuroinflammatory disorders, thus suppression or activation of these selected immune pathways may propose new therapeutic approaches.

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Abbreviations

(RAG)-1	Recombination activating gene
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
COVID-19	Corona virus disease 2019
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
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

Over the past few decades, the established dogma in which the central nervous system (CNS) is an immune-privileged tissue has undergone a significant paradigm shift as innovations in the field of neuroimmunology and psychoneuroimmunology revealed an interplay between the immune system and CNS homeostatic processes, functions, and pathological states [1–3]. Thus, the immune system contributes to the sculpting of brain circuitry, regulation of neuronal communication, and coordination of neurodevelopmental and aging processes. Moreover, increasing evidence suggests that the immune system plays a crucial role in neurodegenerative diseases, neuropsychiatric disorders, the peripheral nervous system, and neuro-oncological conditions [4]. Therefore, investigating neuroimmune interactions could accelerate breakthroughs in the field of neuroimmunology by providing novel insights into nervous system development, homeostasis maintenance, and neurological disease progression. In addition, to open potential new therapeutic avenues for neuroimmune pathological disorders such as multiple sclerosis, Alzheimer's, Parkinson's, chronic depression, schizophrenia, autism, and ataxia to name just a few.

The cerebellum, a small structure of the CNS well-conserved across evolution, is estimated to represent 5–6% of neonates and 11% of human adults' brain weight. Despite its small size, the cerebellum contains approximately 80% of all neurons and plays a crucial role in sensorimotor control and regulation of emotional and higher cognitive functions [5, 6]. Growing evidence associates the cerebellum with satiation center, visuospatial, verbal, memory, and executive functions in addition to suggest its importance in achieving and improving newly learned skills [7, 8]. Hence, functional clinical studies revealed the importance of the cerebellum in development and learning processes during early developing age which ranges from

the first trimester of pregnancy to 2 years postnatal, in childhood, adolescence, and adulthood [6, 7, 9].

In recent years, although limited, several studies have proposed a role for immune-mediated mechanisms in the etiology of several pathological cerebellar disorders such as multiple sclerosis, neuropsychiatric systemic lupus erythematosus, paraneoplastic cerebellar degeneration, nonparaneoplastic immune-mediated cerebellar ataxia, primary autoimmune cerebellar ataxia (PACA), and acute cerebellitis.

The CNS and more specifically the cerebellum and the immune system can interact through immune mediators including reactive oxidative stress (ROS), neurotrophic factors, cytokines, neurotransmitters, and neuropeptides as well as peripheral immune cell infiltration [10]. Although bidirectional communication between the immune response and the CNS and their impact on the cerebellar is yet to be fully understood, direct bidirectional projections between the cerebellum and hypothalamus have been demonstrated [11–13] [14]. Moreover, evidence has shown that the hypothalamus participates in the regulation of the CNS's immediate response to inflammatory stimuli, implying a possible indirect role for the cerebellum in the regulation of immune cell functions through the cerebellohypothalamic projections [15]**.** 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 understanding 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 [16, 17]. For example, histamine-containing fibers originate from the tuberomammillary nucleus of the hypothalamus and broadly spread into the cerebellum [17]. Moreover, beaded fibers containing angiotensin II result from the paraventricular and supraoptic nuclei of the hypothalamus [18] 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 [19]. 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 thyroid hormone during the first two postnatal weeks. In perinatal hypothyroidism, Purkinje cell dendrites have significantly reduced growth and branching with a reduction of synapses between granule cells and Purkinje neurons, which is associated with delayed migration of granule cells precursors to the granule cell layer and deficient synaptic connectivity within the cerebellar cortex [20]. Experimentally, thyroid-deficient rats show a delay in the disappearance of somatic spines, the synaptic site for climbing fibers, along with underdevelopment of cerebellar glomeruli [21]. 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 [22]. 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 development of the cerebellum.

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 direct reciprocal projections between the cerebellum and the hypothalamus and this pathway may serve as an important mediator in immune system modulation. Moreover, many neuropeptides can be released from the CNS and can impact the immune system, which in turn affects the cerebellum, especially in the developmental stages. Thus, various immune responses can shape the development and functional activities of the cerebellum (Fig. 1). 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. 2a, the innate and adaptive immune responses, which lead to the production of cytokines, can alter cerebellar development and function. Moreover, pattern recognition receptors and innate and adaptive immune responses play a key role in the regulation of the immune system.

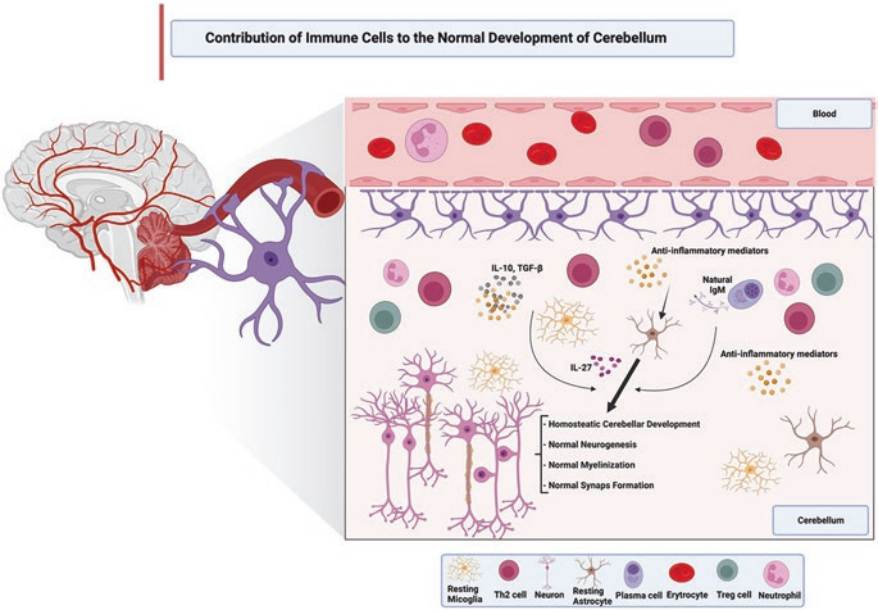

Fig. 1 An illustration of how immune cells contribute to normal cerebellar development. These cells maintain neuronal function and development homeostasis by secreting anti-inflammatory cytokines and regulating the activation status of microglia and astrocytes in local tissues. Microglial cells at rest survey the cerebellum parenchyma and promote a healthy environment for cerebellar development. B cells secrete natural antibodies and promote axon myelination to maintain tissue homeostasis. Resting astrocytes also play a role in maintaining synaptic formation and activity by releasing interleukin (IL)-27 to reduce inflammation and exert neuroprotective effects. Tumor growth factor (TGF), T helper (Th), Immunoglobulin (Ig). (Created with BioRender.com)

Fig. 2 (continued) resulting in abnormal cerebral development. Microglia and complement proteins are critical to synaptic pruning and scaling, whereas brain-reactive autoantibodies can affect the development or function of neurons, including Purkinje cells. (**b**) An illustration of how immune cells interact with cerebellar cells such as astrocytes and microglia during neurodegeneration. There is a cerebello-hypothalamic interaction in normal conditions via the balance of gamma-aminobutyric acid (GABA) and glutamate, which is impaired in pathological conditions such as stress and infection. This imbalance would result in increased cerebellar cell death and the production of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), both of which can activate neurons via their pattern recognition receptors (PRRs). These activated cells send damage signals to glial cells and astrocytes, causing the blood-brain barrier to be breached, resulting in the activation of innate and adaptive immune responses, the production of pro-inflammatory cytokines, and neuro-inflammation. On the other hand, anti-inflammatory cytokines result in the deactivation of glial cells and astrocytes, thereby maintaining homeostasis. (**c**) Role of the bidirectional microbiota-gut-brain axis in neuroinflammation and developmental disorders. This axis primarily acts through microbiota metabolites, which can be absorbed and transported by the blood before crossing the blood-brain barrier to modulate cerebral functions. Dysbiosis of the gut microbiota can lead to cerebral developmental disorders such as autism by modulating the host's immune response in a way that releases pro-inflammatory cytokines. Interleukin (IL), interferon (INF), tumor growth factor (TGF), tumor necrosis factor (TNF). (Created with BioRender.com)

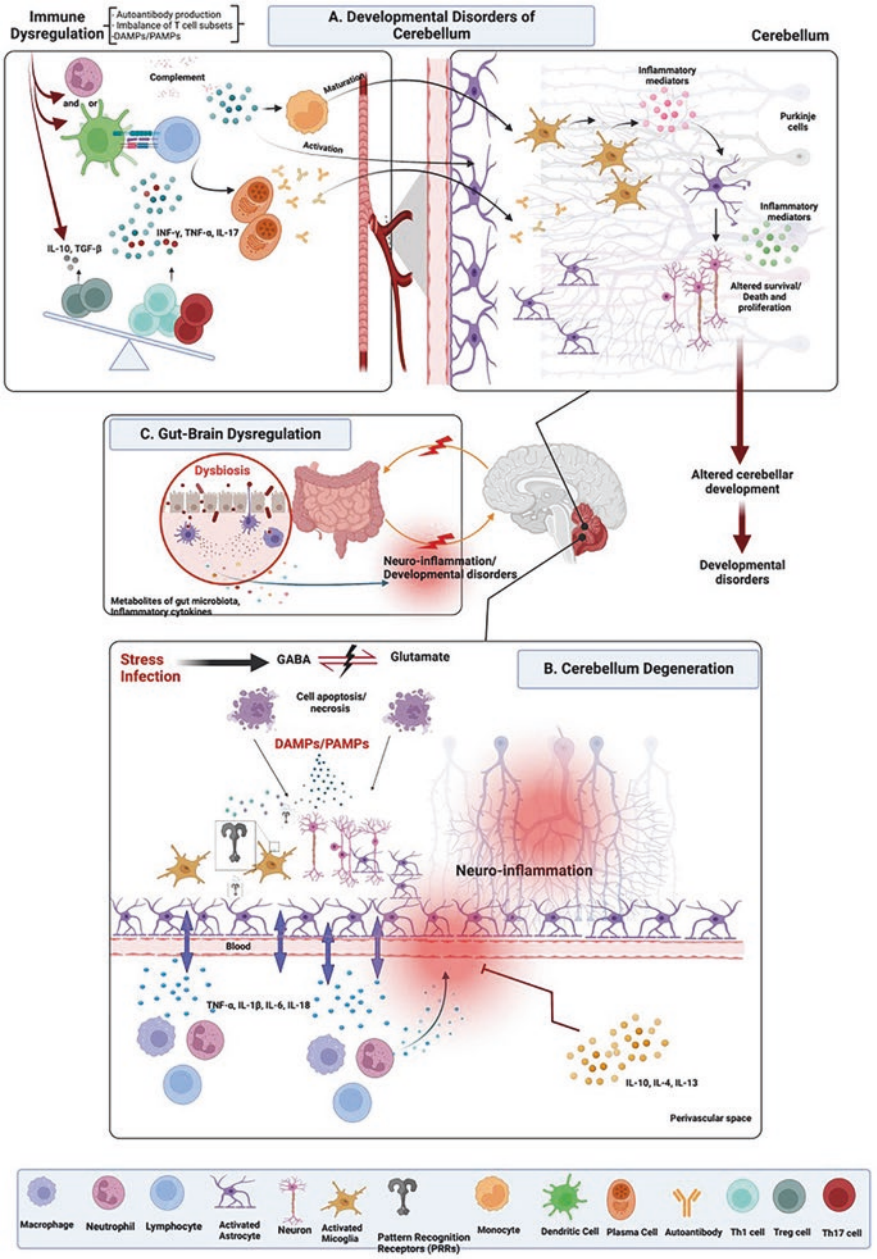

Fig. 2 (**a**) An overview of immune dysregulation in cerebellar developmental disorders. As illustrated, several innate and adaptive immune cells and their immune factors, such as cytokines and complement, are involved in cerebellum development and function. In the presence of altered T-cell subpopulations, autoantibodies and inflammatory cytokines may be produced. Microglia and astrocytes are subsequently activated, impairing neuron survival, proliferation, and death,

(continued)

Pattern Recognition Receptors

The innate immune cells are equipped with germline-encoded pattern recognition receptors called pattern recognition receptors (PRRs) that directly and specifically recognize conserved molecular structures named pathogen-associated molecular patterns (PAMPs) expressed by microbes [23]. Moreover, PRRs play a critical role in sterile inflammatory responses that arise from endogenous stimuli resulting from the release of molecules named damage-associated molecular patterns (DAMPs) following tissue damages. Thus, PRRs are among the first responders to cerebellar disorders [24], and activation of these receptors on microglia, neurons, and astrocytes initiates an innate immune response [25] (Fig. 2b). Microglia, the brain's primary resident immune cells, are fundamental components of host innate immunity in the cerebellum. Distress signals released from neighboring cells can bind to PRRs and activate microglia [26]. Activated microglia enter a pro-inflammatory state and release inflammatory mediators that help in the clearance of antigens and restore tissue homeostasis. However, chronic or continuous activation of these receptors can lead to inflammatory responses that can impact the cerebellum's development and contribute to the pathogenesis of cerebellar developmental disorders [27]. Toll-like receptors (TLRs) are a class of PRRs encompassing several members including TLR1-TLR10 in humans and TLR1-TLR9 and TLR11-TLR13 in mice [28]. Over the last decade, in addition to reporting the expression of all TLRs members in major CNS cells type, new data indicate that upon activation, these PRRs can exert a beneficial or detrimental role depending on the strength and timing of the stimuli, CNS microenvironment, and TLR members activated [29]. In addition to a well-established role in host defense immunity, TLR may also play a central role in regulating sterile inflammation, cell migration and differentiation, tissue development, and CNS repair process following trauma [29, 30]. Hence, during transient focal cerebral ischemia, for instance, TLR4 expression on cerebral endothelial vasculature increases and has been associated with a control in neutrophil recruitment. Moreover, together with TLR2, it has been implicated with exacerbated neuroinflammation and neuronal death [31, 32] (Fig. 2a, b). 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 [33–35]. Mitochondrial DNA and proteins are also considered to be DAMPs, particularly mitochondrial DNA and N-formyl peptides [36].

Most cells in the CNS express TLRs, but microglia express the full repertoire of TLRs, which enhances their ability to monitor the CNS and act as the first line of defense [37]. In the cerebellum, microglial activation can be partially explained by aberrant expression of TLRs in regions of the brain involved in striatonigral (SND) or olivopontocerebellar atrophy (OPCA) [38]. TLRs' inappropriate expression and altered signaling could promote neurodegenerative disorders (Fig. 2b) through the amplification of pro-inflammatory cytokines release which could result in mitochondrial dysfunction and excessive ROS production [38]. Astrocytes, neurons, and oligodendrocytes also express TLRs in both physiological and pathological

cerebellum states [39]. Astrocytes express TLR3 under resting and activated conditions [40] and may elevate TLR2 and TLR4 upon activation [41]. 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 [42]. 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 [43], and for programmed cell death [44]. Within the CNS, three different NLR inflammasomes have been described: NLRP1 [45], NLRP2 [46], and NLRP3 [47]. NLRP1 has been defined as mediating the innate immune response after brain disorders [48]. Inflammasomes are present in astrocytes [49] and microglia [50], and neuronal inflammasomes seem to contribute to cerebellum disorders [51]. IL-1β and IL-18 have a crucial role in mediating neuroinflammation and neurodegeneration in the CNS [52]. 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 [52]. IL-1β also participates in neurological processes and appears to have a role in autism as a mediator of this cerebellar developmental disorder [53]. Homeostatic levels of IL-1β and its antagonist IL-1ra are necessary for proper brain development and function.

Many PRRs expressed in the cerebellum can identify pathogenic microbes and are mediated through RLR and ALR [54]. RLRs are cytoplasmic PRRs that detect RNA viruses associated with the production of type I interferons (IFNs) [55]. Two ALRs have been described: IFI16 and AIM2 [56]. In neurons, AIM2 forms an inflammasome that activates pyroptosis, a novel but potentially important mode of cell death [57]. 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 [58]. Programmed cell death plays a significant role in 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 CNS/cerebellum have passed from being defined as an immune privilege system to a special immune-controlled site mediated by resident immune cells, microglia, and astrocytes which comprised most brain immune cells. Classical immune cells such as myeloid, monocyte/macrophages, natural killer

(NK), B, and T cells have also been identified in CNS at a steady state [59]. Although scarce, resident classical immune cells are shown to have a major influence on brain function. Hence, T cells for example have been implicated in adult brain neurogenesis and cognitive function including spatial learning, memory, emotional behavior, and response to stress. Whereas B cells are highly expressed in neonate's brain and participate in the maintenance of tissue homeostasis by secreting natural IgM antibodies and promoting the proliferation of oligodendrocytes which are known to participate in axons myelinization [59]. Interestingly, several reports suggested a potential pathogenic role for blood-derived immune cells such as neutrophils, lymphocytes, and myeloid cells in neurodegenerative and neuroinflammatory disorders such as Alzheimer's diseases, multiple sclerosis, and ischemic stroke following the disruption of the blood-brain barrier [60]. Moreover, microglia, dendritic cells (DCs), and astrocytes are also implicated in significant crosstalk 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 (APC) that induce adaptive immunity (Fig. 2a). 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 [61]. 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 [62, 63]. The parenchymal cerebellum environment has anti-inflammatory proprieties because of high local levels of inflammation-suppressive cytokines (TGF-β, IL-10) (Fig. 2b) and it is supplied with gangliosides, which can be detrimental and lethal to T cells [64, 65]. 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 [25].

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 [66] (Figs. 1 and 2). Microglia are implicated in several functions including the growth of neurites, synaptic pruning, spinogenesis, and apoptosis [67, 68] in areas such as the visual cortex,

hippocampus, and retinogeniculate system, during brain development [69]. 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 [70]. Recent findings showed a continuous process of microglial maturation and a non-uniform distribution in the cerebellar cortex, demonstrating that microglia are an essential cellular component of the cerebellum [71]. This has been confirmed *in vitro*, where microglia have been shown to promote apoptosis of Purkinje neurons [72]. 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 [73]. 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 [74].

Neutrophils are a key 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 [75]. Neutrophils respond to PAMPs and DAMPs through TLRs and NLRs to increase CD15, CD11b, and adhesion molecules expression, which are responsible for neutrophil recruitment [76] (Fig. 2a, b). Activated neutrophils release inflammatory mediators, angiogenic factors, lytic enzymes, and antimicrobial peptides [76], and play a critical role in Th1 or Th17 recruitment through the production of CXCL9, CXCL10, or CCL20 [77, 78]. Neutrophil-lymphocyte interactions release survival factors that increase the lifespan of the short-lived neutrophils [25].

Chemokines and their receptors (CCL2/MCP-1, CCL5/RANTES, CXCL12/CXCR4) have an important impact on the development and maintenance of the cerebellum [79], and they are expressed in several parts of the brain including the cerebellum [80]. Moreover, chemokines may influence the crosstalk between neuron and glial cell types and can function as a third communication system in the brain [81]. 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 or external germinal zone (EGZ)) 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, exit from the cell cycle, differentiate, and radially migrate via the Purkinje cell layer to their final destination [82]. CXCL12, a strong chemoattractant for granule cell precursors in the URL and EGL, also plays a critical role during neurogenesis through the promotion of axonal growth and is expressed in embryonic and postnatal meninges that cover the cerebellum [83]. It is also known as a potent chemoattractant for URL cells, inhibiting CXCR4-expressing premature granule cell migration to the EGL [84]. 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 the differentiation and homeostasis of neurons and influence synaptic activity. They are also responsible for the formation of the BBB [85] (Fig. 2a, b). 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 [86]. The existence of numerous astrocytic end-feet near the BBB demonstrates their role in the 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 [87]. 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 [88]. Following PRR activation, astrocytes produce cytokines, chemokines, and neurotrophins that target neighboring glial cells and neurons [88]. 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 [89]. Astrocyte activation leads to activation of damage control mechanisms such as induction of a neuroprotective effect and polarization toward the Th2 profile. Conversely, IFN-γ produced by Th1 cells can suppress astrocytes that aggravate neuroinflammation [90]. Thus, astrocytes may constrain and defer neuroinflammation, but elevated levels of IFN-γ might promote astrocytes to become potent APCs and even promote inflammation [91].

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 [92, 93]. The liver is the main source of complement production, but it can be produced by many types of resident cells in the CNS [94]. Complement receptor expression (C3a and C5a) has been shown on glial cells and neurons [95] (Fig. 2a). The complement system contributes to modulating CNS development and inflammation [96]. Complement components C1q and C3 are expressed on neurons throughout the CNS where they opsonize synapses to highlight them for phagocytosis by microglia [97]. Their expression peaks during crucial stages of neurodevelopment such as synapse formation and activity-dependent refinement [98]. 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 [99]. Systemic complement depletion diminishes perihematomal brain edema and TNF-α release following experimental intra-cerebral hemorrhage [100]. 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 [88]. 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 Cerebellum

Adaptive immunity is orchestrated by T-helper (Th) cell subsets, through the 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 (Fig. 2a). 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 [101]. Conversely, the suppressive influence of vestibulocerebellar lesions on immune function demonstrates 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 [102]. Subsequently, cerebellar fastigial nuclei contribute to the modulation of lymphocyte function but not to the hypothalamic-pituitary-adrenal axis [102].

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 [103]. 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 [104]. Moreover, regulatory T cells (Treg cells) reduce microglial activation after inflammation develops, and astrocytes promote Treg cell transcription factor expression [105]. Therefore, T cells are key players and might have a beneficial role in the development of CNS adaptive immunity (Figs. 1 and 2).

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 [106, 107]. 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 ($ROR\gamma t^{+}$, $T\text{-}bet^{+}$, $GATA\text{-}3^{+}$) [108]. 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 (Fig. 2a). The immune system plays a crucial role in the recovery process of cerebellum development and disorders [109]. 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 [109] (Fig. 2a). 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 [110]. 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, a class of GABAergic neurons located in the cerebellum, have the potential to shape adaptive immunity. Immunoglobulin plays a role in many neurodisorders. Antibodies to cytoplasmic components of Purkinje cells have frequently been labeled in serum and CSF [111]. 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 [112]. The cerebellar Purkinje cell is a possible exception. Experimentally, Purkinje cells showed high endocytic activity for a wide range of substances that originate from the ventricular CSF [113], and they can also incorporate IgG and S100 [111]. 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 [111].

Cerebellum and Immune Response Interactions in Cerebellar Diseases

Cerebellum and particularly Purkinje cells seem to be a common immunological target in some neurological disorders (Fig. 2a). 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 [114]. 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) [115]. Many of these autoantibodies distinguish cerebellar-specific antigens traced in the Purkinje cell soma to dendrites resulting in a Medusa-head immunohistochemical staining pattern [116]. 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 (Fig. 2c). Examination of patients with gluten ataxia revealed patchy loss of Purkinje cells in the cerebellar cortex [117]. 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 [117]. Similar findings have been defined in patients with established celiac disease who then developed cerebellar ataxia [118]. Experimentally, antibody cross-reactivity between antigenic epitopes on Purkinje cells and gluten peptides has been reported [119]. 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 anti-gliadin 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 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 [120]. 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 [121]. Overall, 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 (Fig. 2c), which is regarded as the gut-brain axis, is a well-known bidirectional neuro-humoral communication system. Previous research that focused on the gut-brain axis mostly referred to its contribution to functional gastrointestinal syndromes, such as irritable bowel syndrome (IBS) [122]. It was recently reported that gut microbiota can modulate brain development and produce behavioral phenotypes via the gut-brain axis [123]. 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 microbiota also contributes to cerebral developmental disorders by modulating the host immune response by releasing a storm of pro-inflammatory cytokines (including IL-1, IL-6, and IL-18) by intestinal epithelial cells, intestinal DCs, and macrophages [124]. 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 [125]. 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 extra-intestinal sites such as the CNS and or cerebellum [126]. IgA deposition on jejunal tissue transglutaminase has been reported in the jejunal tissue but also in the brain (mostly in the cerebellum) of patients with gluten ataxia and in none of the controls [127]. 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) [117]. 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 the 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 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 key role physiology of cerebellar ataxia because celiac disease is caused by an exogenous protein antigen and is linked to HLA DQ2/8 expression.

Neurological Disorders Associated with Coronavirus Disease 2019

Viral infection has long been known to cause neurological complications resulting in neurodegenerative disorders [128, 129]. Among neurotropic viruses, in addition to well-known enterovirus such as poliovirus (poliomyelitis), respiratory viruses including influenza virus (flu), flavivirus (Zika), and coronaviruses (severe acute respiratory syndrome, SARS-CoV, and Middle Eastern respiratory syndrome, MERS) to name but few, have been shown to invade the CNS and infect CNS cells causing short- and long-term neurological sequelae [129].

Recently, a new coronavirus infection, SARS-coronavirus-2 (SARS-CoV-2), responsible for the coronavirus infectious disease 2019 (COVID-19) pandemic which started in late 2019, has spread worldwide [130]. Up until now, more than 380 million COVID-19 confirmed cases have been recorded and retrospective studies from around the world report debilitating neurological manifestation in more than a third of severely ill COVID-19 patients [131–133]. Thus, emerging data from case reports described acute neurological symptoms including ischemic strokes, encephalopathy, and encephalitis and chronic neurological sequelae such as fatigue, ataxia, neurocognitive impairment along with visuospatial and executive dysfunctions in patients with COVID-19 [131, 134, 135]. SARS-CoV-2 infection is mediated by the engagement of its spiked (S) protein to the angiotensin-converting enzyme 2 (ACE2) receptor [130]. ACE2 receptors are not only present on the surface of alveolar and respiratory epithelial cells but they are also highly expressed in CNS tissues and cells including neurons, astrocytes, cerebral blood vessel, thalamus, and the cerebellum [129, 134]. Although the mechanism associated with neurological disorders in COVID-19 patients remains speculative, multiple potential pathogenic processes have been suggested and are currently under investigation. One of the proposed mechanisms associated with COVID-19 neuropathologies includes the invasion of the CNS by SARS-CoV-2 through ACE2 receptors which may result in viruses' direct actions on neurons and astrocytes [136]. Secondly, SARS-CoV-2 have been shown to induce a systemic inflammatory response accompanied with cytokines storm and increase BBB permeability and microvascular injury [134]. Disruption of the BBB integrity may result in SARS-CoV-2 and host-activated immune cells entering the CNS and accentuating further neuroinflammatory response and tissue damage [134]. While the microvascular injury may likely lead to thrombotic and microhemorrhage events as described in seriously ill COVID-19 patients [134, 137]. Based on our current knowledge, more investigation will be required to improve diagnosis and prevent potential long-term COVID-19 neurological disorders.

Diagnostic Tests and Managements of Immune-Mediated Cerebellar Disorders

Cerebellar disorders encompass a heterogeneous group of pathological conditions that result from various etiopathology. Hence, cerebellar dysfunction can arise from genetic defects, metabolic deficiencies, neurodegenerative diseases, toxins presence, structural lesions caused by tumor, trauma, or stroke, and immune-mediated cerebellar insults, which can lead to a myriad of sensorimotor, affect, and cognitive disorders along with autonomic nervous system problems [138, 139]. Therefore, to discover the underlying pathophysiological mechanisms during the cerebellar exam of an ill patient, cerebellum disorders are first broadly categorized into four distinct groups: genetics, non-genetic acquired conditions, sporadic neurodegenerative, and idiopathic late-onset cerebellar ataxia (ILOCA). Several elements including motor and non-motor symptoms, family history, risk factors (e.g., alcohol consumption, infection, multiple sclerosis), and diseases progression are investigated to reach an accurate diagnosis [140].

According to the differential diagnosis based upon the initial assessment, diagnostic tools such as brain magnetic resonance imaging (MRI), CT or PET scan of the whole body, genetic analysis, and blood chemistry can be performed in search for disorders caused by structural abnormalities, occult primary tumors, genetic or metabolic defects [138, 140–145]. Moreover, serum and cerebrospinal fluid testing are indicated to identify the presence of bacterial, viral, and parasitic infections such as Lyme, tuberculosis, Whipple, varicella, HIV, toxoplasma, and malaria as well as onconeuronal antibodies (e.g., Hu, Yo, Ri, Ma, TA, CARP8, CV2, Tr, LEMS, MGLUR1, CRMP5, GQ1b, amphiphysin, PCA-2, NMDA, VGKC) and autoantibodies anti-GAD and gluten, all known to trigger immune-mediated cerebellar neuropathologies [138, 140–144]. These testings also permit the segregation between all immune-mediated cerebellar disorders subtypes including gluten ataxia, cerebellar degeneration, post-infection cerebellitis, and anti-GAD ataxia [138].

Despite the rapid progress in research on cerebellum development, functions, and pathologies in recent years, the treatment of cerebellar disease remains clinically challenging owing to the various neurological and systemic disorders that may affect the cerebellum.

Hence, cerebellar diseases therapeutic strategies largely depend on the underlying pathological causes and are based on measures such as rehabilitative therapies (see Chapter "Rehabilitation in Cerebellar Ataxia"), eliminating toxins, or remediating to metabolic deficiencies. In the case of immune-mediated cerebellar diseases initiated by gluten sensitivity, tumors, or infections, treatments focus mainly on eliminating these primary pathological conditions. However, in the absence of other underlying pathologies, at the source of the immune-mediated cerebellar disorders, immunotherapy treatments are immediately prescribed to control the immune response. The regimen of these immunotherapy treatments includes corticosteroids such as methylprednisolone and prednisolone, which are administrated intravenously or orally to induce and maintain remission in patients with immune-mediated cerebellar diseases.

Moreover, additional strategies such as intravenous immunoglobulin (IVIg), plasmapheresis, monoclonal antibodies treatment against B cell CD20 proteins Rituximab, and immunosuppressants drugs cyclosporin, mycophenolate mofetil, and cyclophosphamide, that selectively inhibit B and T cells activation and proliferation, can be utilized to suppress the immune response.

Conclusion

Understanding the links between the immune, CNS, enteric, and endocrine systems is fundamental to understanding the bidirectional communication between the immune system and cerebellum. An imbalance in the neuro-immune interaction may promote the onset of autoimmune disorders and constitute a key component of pathogenic mechanisms involved in neurodevelopmental (Fig. 2a) and neurodegenerative diseases (Fig. 2b) such as autism and cerebellar ataxia. 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

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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 behavioural 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 effects of 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 the causes, mechanisms, and manifestation 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

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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 medication such as phenobarbital, diphenylhydantoin, valproic acid; retinoic acid; warfarin; etc.); heavy metals and 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.); 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; 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, 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 behavioural 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 injury due to a variety of toxins results in neurologic deficits, including ataxia, hypotonia, dysarthria and ocular motility problems. This can present with impairments in movement, motor coordination, and sensory function, cognition and affect regulation or mood. Dysfunction of the cerebellum and its effects on connectivity to other brain regions has been correlated with a number of neurodevelopmental disorders that include autism, attention deficit hyperactivity disorder, dyslexia, as well as psychiatric diseases 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 sub-regions of the cerebellum involved and determined by alterations in the corresponding cerebrocerebellar 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 such therapeutic agents as 13-cis retinoic acid (Accutane©) and misoprostol (Cytotec©) [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 map 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" (***T****oxoplasma 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 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 the 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 [16, 23, 24]. Further investigations could clarify the role of viral infections'over-stimulation of excitatory amino acid receptors, excess production of angiogenesis, pro-inflammatory cytokines 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 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's [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 the 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 which are on drug therapy for management of this common disorder [42]. This is a concern since almost all antiepileptic drugs have potential risks for 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, 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 anticonvulsant 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 during 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 centres using a prospective, observational, registration and follow-up approach. They found 4.2% of women delivered

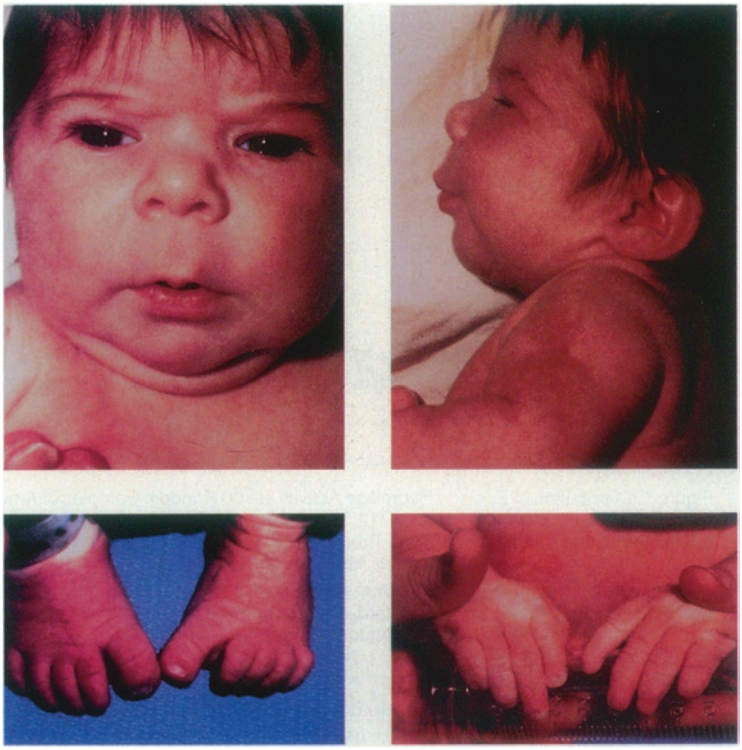

Fig. 1 Infant with typical facial features and distal digital hypoplasia with fetal hydantoin syndrome from Buehler et al. NEJM 1998, needs permission (with permission)

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 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, levetriracetam, topiramate, vigabratin 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 2–3 times that of the expected rates of malformations in the population, and is associated with a higher risk than 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 bida 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 the fetal hydantoin syndrome and confirmed reduced activity of epoxide hydrolase in those exposed 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 Spectrum 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 the irrefutable evidence of the harmful effects of alcohol in pregnancy becomes 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 behavioural 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 behavioural 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 [79–85]. The term fetal alcohol spectrum disorder has often been used to include the whole

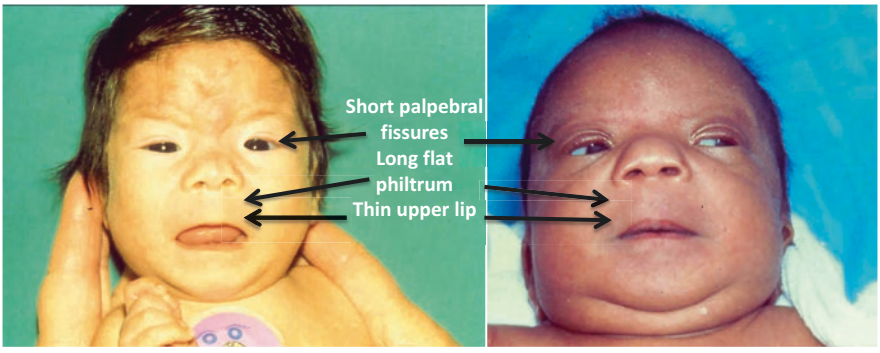

Fig. 2 The typical facial features of fetal alcohol syndrome in two infants

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 behavioural, 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 paediatricians, 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, and 5. executive function (including impulse control and hyperactivity, adaptive behaviour, social behaviour, social skills or social communication, attention, affect regulation) 6. 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 co-morbid 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-spatial 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 with 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 is 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 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 birth [106], consistent and nurturing child care, early diagnosis with earlier interventions, and favourable genetic factors (particularly those involved in alcohol metabolism). According to May and Gossage [107] maternal risk is multidimensional, including

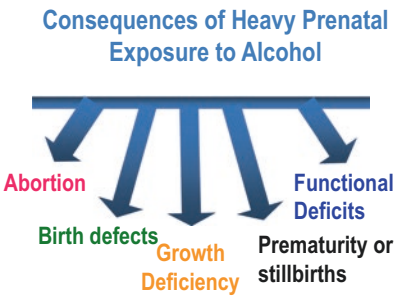

Fig. 3 Variable fetal outcomes from excessive ethanol exposure

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 or emotional or sexual abuse, repeated changes in caregivers and place of residence, "unfavourable" 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 4167 children were strongly related to lower IQ at age 8, as was a risk allele score based on these 4 variants. All the mothers of these children took moderate amounts of alcohol during the pregnancy. The authors suggest that, even amongst women drinking moderate amounts of alcohol, subtle changes in exposure to alcohol due to an ability to metabolize the

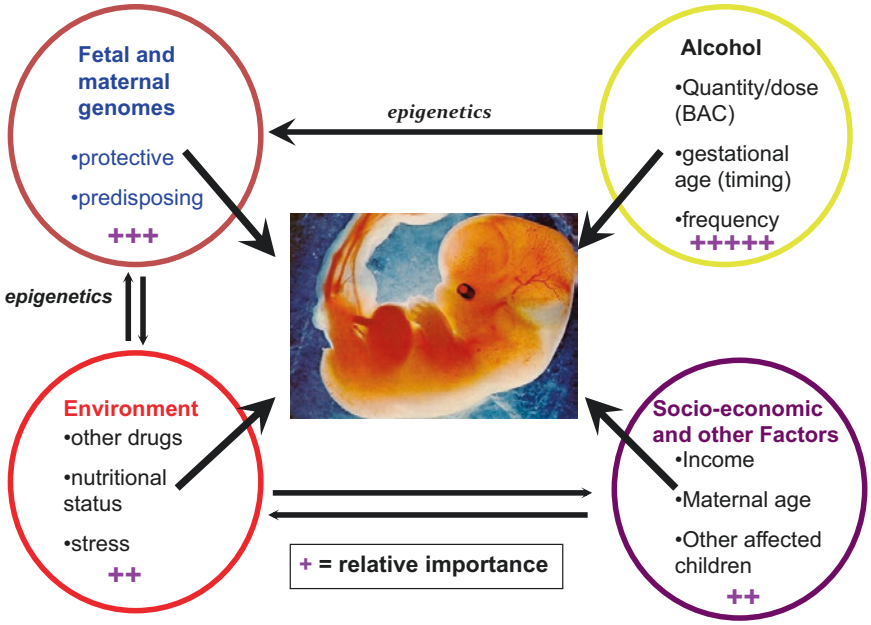

Fig. 4 A schematic representation of risk factors contributing to FASD

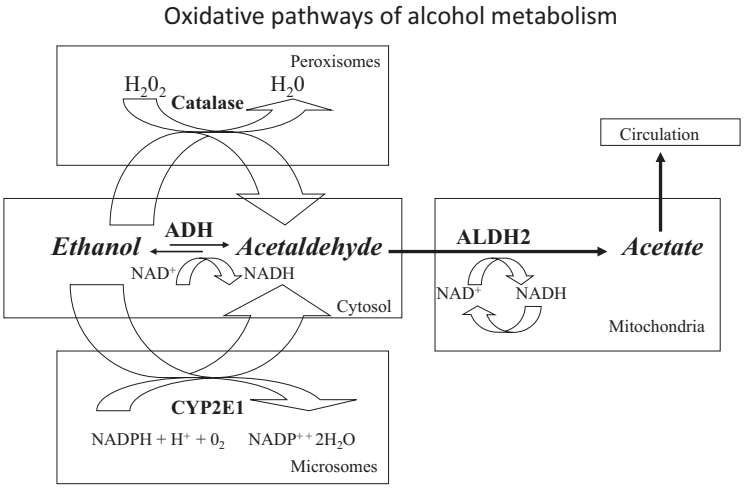

Fig. 5 Oxidative pathways of alcohol metabolism. The enzymes alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1) and catalase all contribute to oxidative metabolism of alcohol. ADH, present in the fluid of the cell (i.e. cytosol), converts alcohol (i.e. ethanol) to acetaldehyde. This reaction involves 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 (H_2O_2) 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 AE**.** Genetic factors in Fetal Alcohol Spectrum Disorder. In Fetal Alcohol Syndrome Disorder. Management and Policy Perspectives of FASD. E Riley, S. Clarren, J. Weinberg, E. Jonsson, New York, Wiley/Blackwell, 109–126, 2011. Needs permission)

substrate may be important, and offers 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 signalling [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 signalling controlling cell survival or death (growth factors deprivation, oxidative stress, apoptotic signalling and caspase-3 activation, suppression of NMDA glutamate and GABAA receptors, withdrawal-induced glutamatergic excitotoxicity) [121, 122]; (5) 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 over-represented among the top-ranked candidate genes. These pathways include the TGF-β signalling pathway, MAPK signalling pathway and the Hedgehog signalling pathway.

The genes in the TGF-β signalling 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 signalling pathway an important consideration, as it is essential in fetal and CNS development. Alcohol inhibits such TGF-β regulated processes 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 signalling pathway interacts with alcohol, and/or its metabolic breakdown products, and that 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 post-mitotic functions, and in cell differentiation. The MAPK signalling 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 signalling pathway was also identified to contain several genes within the candidate list. This signalling pathway is a highly conserved and key regulator of embryonic development. Knock-out 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 signalling components, specifically the sonic hedgehog (Shh) molecule [130–132]. 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 signalling. 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 signalling 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 tumour 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 were analysed (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 aetiology 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 (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, and 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 Purkinje cell counts in an early-maturing region (lobules I-X) and a late-maturing

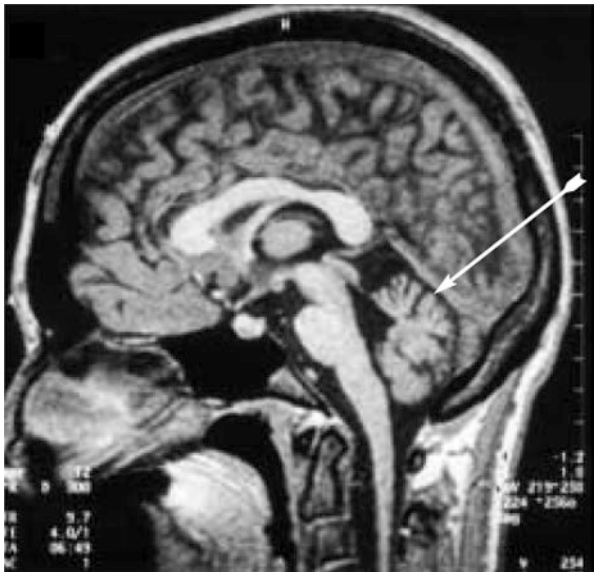

Fig. 6 An MRI demonstrating a small cerebellum and vermis hypoplasia (arrow) in a child with FAS. (From fig. 1 in Autti-Rämö I, Autti T, Korkman M, Kettunen S, Salonen O, Valanne L. MRI findings in children with school problems who had been exposed prenatally to alcohol. Dev Med Child Neurol. 2002 Feb;44(2):98–106.) Needs permission)

region (lobules VIc-VII) from mid-sagittal 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 post-mitotic 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) signalling [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 signalling 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 showed that reduced N-acetylaspartate NAA levels in children with PAE using MRS suggest impairment in the early developmental formation of dendritic arborizations and synaptic connections [176]. The study showed 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 were 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 analysis. Using DTI (which is used to assess the 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 fibres 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 behavioural 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 longstanding recognition and association with cognitive function and cerebellar function [178–181]. Behavioural 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 the boys with ADHD. This reduction involved mainly the posterior inferior lobe (lobules VIII to X) but not the posterior superior lobe (lobules VI to 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 over-representation 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's 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 in the recognition of aetiology, mechanisms of teratogenesis, pathogenesis and clinic 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 an 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 (ATRT), pilocytic astrocytomas and ependymomas. In the companion 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 5th edition of the World Health Organization (WHO) classification of Tumors of the Central Nervous System (Louis et al. Neuro-Oncology. 2021;23(8):1231–51, 2021).

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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, [115]). 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 (ATRT), 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

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Table 1 Posterior fossa mass: differential diagnosis

Pilocytic astrocytoma
Medulloblastoma
Ependymoma
Atypical teratoid/rhabdoid tumor (AT/RT)
Brainstem glioma
Metastatic deposits
Hemangioblastoma
Teratoma
Dermoid cyst
Meningioma
Vestibular schwannoma
Lymphoma
Ganglion cell tumor/ganglioglioma
Lhermitte-Duclos disease

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), non-WNT/non-SHH (WHO CNS 52021)	PFA: Epigenetic aberration PFB: 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 II, III, myxopapillary, subependymoma, 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, chemotherapy/targeted therapy for progressive disease; radiation is rarely indicated
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 to 11 months	10-year overall survival rate > 90%

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 molecular genetic signatures facilitated more recently by tumor DNA methylation-based classification [19]. This has important implications for the selection of future molecular targets and new therapeutic intervention strategies.

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 [52, 145]. These brain tumors have historically been characterized by their aggressive behavior and poor prognosis, with a median survival ranging from 6 to 11 months [23, 74, 100, 136]. ATRTs are the most common malignant CNS tumor affecting children younger than 6 months of age [39]. Approximately 70% of cases arise in children younger than one year of age, and 90% occur before three years of age [60], with a median age of 18 months [42].

Overall, ATRTs are estimated to comprise 1–3% of pediatric brain tumors [77, 82], but they account for 20% of CNS tumors in children under the age of 3 years [77]. 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 [98]. Relative survival estimates for embryonal tumors are low, but vary significantly by histology. The current 10-year survival rate for ATRT is 26.5% [98].

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%) [77]. In the

past, ATRT was associated with an extremely poor prognosis, with mean overall survival ranging from 6 to 18 months [100, 141]. 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. In earlier decades, 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 [77].

ATRTs were first described in 1987, but were not recognized as a separate tumor entity by the World Health Organization (WHO) until 1993 [70], when they were classified as an embryonal grade IV neoplasm [71]. ATRT is now defined by alterations of either INI1/SMARCB1 or, very rarely, BRG1/SMARCA4 [48, 66, 156]. 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 and 2021 classifications, 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 [81, 83, 84].

Clinical Presentation

ATRTs arise in infratentorial or supratentorial locations in almost equal proportions, and rarely arise in the spine [8, 74, 150]. 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 [110, 123, 124]. 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 [39].

Imaging features have often been considered non-specific [110, 152]. Parmar et al. [110] 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. [152] 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 [52, 141]. Supratentorial tumors and those affecting both compartments were significantly larger than those in the infratentorial area. 15% 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 were 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% of patients, with an even distribution between the infra- and supratentorial compartments. This feature seems to be a regular finding in ATRTs [152]. 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 [110, 152].

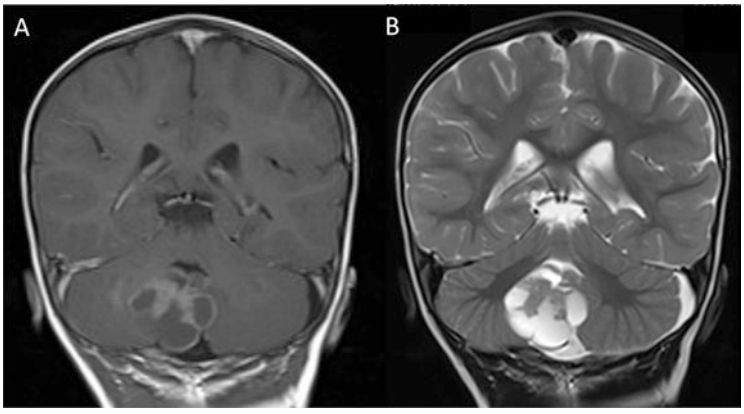

Fig. 1 17-month male with a 3.5 cm atypical teratoid/rhabdoid tumor, localized to the right cerebellar hemisphere with a central solid component and several cystic loculations. (**a**) T1 post-gadolinium. (**b**) T2-weighted image

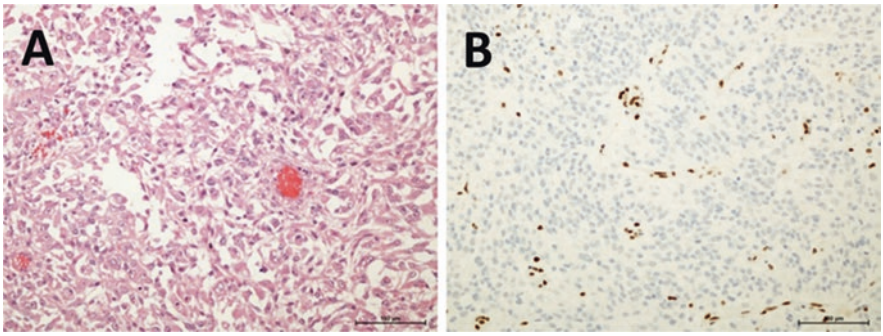

Fig. 2 (**a**) Rhabdoid component of an atypical teratoid/rhabdoid tumor. (**b**) Diagnostic loss of nuclear expression of INI1 in tumor cells, in contrast to INI1 expression in endothelial cells (serve as internal positive controls)

On MRI, signal intensity values on T1- and T2-weighted MR images vary widely [152]. An example is provided in Fig. 1. Parmar et al. [110] 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 [110, 152], 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 [110].

Parmar et al. [110] 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, ATRT are soft, pinkish-red, often well-circumscribed tumors with areas of necrosis and hemorrhages. These tumors arise in the cerebellopontine angle and variably infiltrate the cerebellum and brain stem. ATRTs consist of heterogeneous cells with various morphological appearances [123]. Small undifferentiated embryonal cells are the most common tumor cell population, characterized by high nuclear:cytoplasmic ratio. They often contain a vesicular nucleus, a single nucleolus, and show less nuclear hyperchromasia than cells of other CNS embryonal tumors. Small groups or scattered rhabdoid cells typically with eccentrically placed nucleus, large eosinophilic nucleolus, abundant eosinophilic 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 [89]. 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 ([67]; Fig. 2b) and most other tumors, in particular other CNS embryonal tumors, 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 [50] and is provisionally recognized as a distinct tumor class in the WHO CNS 5 [84]. 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 [61]. 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* [39, 49, 78, 79]. 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 [60, 154]. The SWI/SNF complex has important functions in neural development [31,158]. Using iPSC and cerebral organoid models, it was demonstrated that SMARCB1 loss interacts with neurodevelopmental processes in the process of ATRT tumorigenesis [107].

ATRTs are associated with mutation or inactivation of the *INI1/hSNF5/BAF47* tumor suppressor locus on chromosome 22q11.23 in almost all cases [120, 149]. ATRT is characterized by the biallelic loss of *SMARCB1* expression [123]. 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 [16, 28, 129]. The majority of germline mutations occur de novo, and transmission across generations is rare [3, 41].

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 [69, 78, 79]. Reconciling clinical heterogeneity with tumor biology has been challenging, because it is a rare disease and there have been few biological and clinical studies [17], especially ones which studied CNS ATRT independently from non-CNS rhabdoid tumors [145].

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 [60, 145]. Torchia et al. [145] identified two molecular subgroups of SMARCB1 mutated ATRT with distinct features. Subsequently, Johann et al. [60] 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 [60]. Johann et al. [60] termed these molecular subgroups ATRT-TYR, ATRT-SHH, and ATRT-MYC.

The international consensus following the transcription and methylation profiling studies by Torchia et al. [145] and Johann et al. [60] supports the existence of three different molecular subgroups [53]. From a clinical perspective, Torchia et al. [145] stratified these tumors into average, high, and very high-risk groups by integration of tumor molecular subgrouping and clinical prognostic factors. They defined Group 1 ATRT tumors as those most highly enriched for genes involved in 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 [94, 131]. The *HES5/6* and *DLL1/3* genes, which are also involved in the *NOTCH* pathway, were also highly enriched in group 1 ATRTs [24]. Torchia et al. [145] 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 [145].

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 [145]. 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 [145].

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 to 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 is heterogeneous and represented approximately 40% of cases 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 group led by Kool and Hasselblatt further investigated 65 ATRT-SHH tumors using DNA methylation and t-SNE analysis and identified three molecular subgroups with different clinical, histopathological, and prognostic significance, SHH-1A (younger, supratentorial, less favorable), SHH-1B (older, supratentorial, more favorable), and SHH-2 (younger, infratentorial, less favorable outcomes) [35].

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 embryonal tumors.

A recent study assessed primary cilia which are present in all forms of ATRT. Although the three molecular subgroups demonstrate different patterns of ciliogenesis-associated gene expression, the ATRT-TYR subgroup is particularly enriched. Of potential therapeutic interest, disruption of primary ciliogenesis using SMARCB1-deficient *Drosophila* (*Snr1* gene) or ATRT orthotopic xenograft mouse models resulted in improved survival in these model systems in vivo [14].

Epigenetic-based therapies using newer histone deacetylase inhibitors (HDACi) such as panobinostat may mimic histone acetylation in SMARCB1-deficient tumor cells. Increased neuronal differentiation, decreased tumor growth, and increased survival were evident in vitro and in vivo [25]. Proteosome inhibitors, especially marizomib which crosses the blood-brain barrier, have shown promising results

in vitro and in vivo [93]. A potentially promising preclinical study combined dual mTORC1/2 inhibition using TAK-228 and the BH3 mimetic obatoclax, a potent inducer of apoptosis and oxidative stress in ATRT tumor cells in vitro and in vivo, extending survival in orthotopic xenografts [109]. Furthermore, B7-H3 targeted CAR T cells administered either intracerebroventricularly or intratumorally in cerebral ATRT mouse xenografts; this provides a proof of principle for developing CAR T-cell therapies against this difficult-to-treat tumor in patients [142].

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 [46]. 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 [45, 47, 141]. Small cohorts of patients treated with ATRT-specific regimens have achieved survival rates greater than 50% [23, 141]. Improved survival has also been demonstrated for patients with gross total resection [23, 74].

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 [74, 118]. While a multi-modal 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 [60, 135].

In recent years, treatment approaches in Canada have been more homogeneous and based on the use of high-dose chemotherapy [74]. 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 [8, 23, 44, 74, 141]. The series of patients investigated by Lafay-Cousin et al. [74] 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. ACNS0333, a Children's Oncology Group trial, was the first ATRT-specific study to prospectively evaluate the safety and efficacy of high-dose chemotherapy and 3D-conformal RT. Fifty-four of 65 evaluable patients were <3 years of age; 4-year EFS and OS for the entire cohort were 37% and 43%, respectively [119].

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 [145]. Current

curative therapy for ATRT is perhaps excessively toxic, including the acute toxicity of high-dose chemotherapy [44], 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. [145] 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 [145]. This will have to be validated in future trials. The EU-RHAB registry analyzed 143 patients with clinical, genetic, and treatment data accrued from 2009 to 2017; DNA methylation profiles were available for 84 patients. Negative prognostic factors included: germline SMARCB1 mutation, age <1 year, a non-ATRT-TYR molecular signature, metastatic or synchronous tumors, and omission of RT; however, only age and a non-TYR signature were independent negative prognostic factors for OS [40].

Investigators from the St. Jude Children's Research Hospital's consortium assessed the clinical relevance of ATRT molecular subgroups in 74 patients enrolled on two prospective clinical trials, SBMB03 (ages 3–21 years) and SJYC07 (ages <3 years). Methylation profiling was feasible in 64 patients and demonstrated ATRT-TYR in 21, ATRT-SHH in 30, and ATRT-MYC in 13 patients, respectively. The SHH was associated with metastatic disease. ATRT-TYR was prognostic for better survival in the infant group [148]. 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 [54]. Current targets under consideration are aurora A kinase, cyclin D1, EZH2, and insulin-like growth factor-1. Based upon initial observations by Wetmore et al. [153], the Aurora Kinase A inhibitor Alisertib was evaluated in patients with recurrent/progressive ATRT in a Phase II clinical trial [55, 147]. Of 30 evaluable patients, stable disease (in 8) and partial response (in 1) were seen with PFS of 30% and 13.3% at 6 months and 1 year, respectively, and a 1-year OS of 36.7% [147].

Results from Torchia et al. [145] suggest that inhibitors of *NOTCH, BMP,* and MAPK signaling and angiogenesis may be important novel, subgroup-specific therapeutic agents for ATRT.

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 1 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 [18].

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%), brainstem (9%), and the spinal cord (2%) [18]. 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 was omitted in the 2016 update [81, 83] and the entity was not included in the 2021 update [84].

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). RAPNO (Response Assessment in Pediatric Neuro-Oncology) guidelines for the neuroradiological

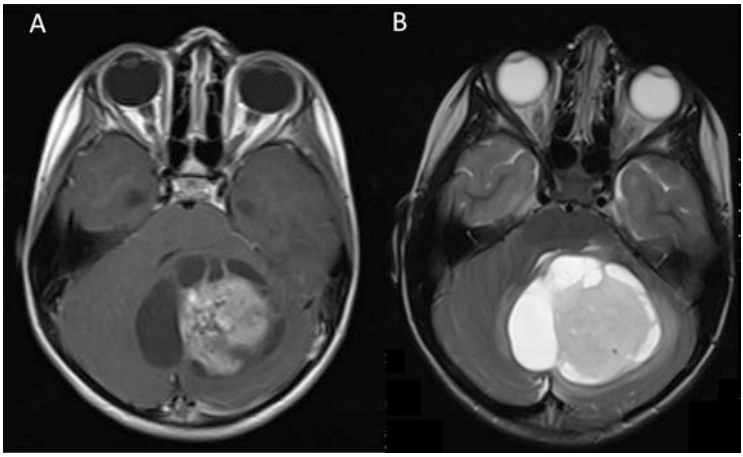

Fig. 3 4-year-old with a large 5.4 × 5.8 × 5.2 cm mass, a pilocytic astrocytoma located in the left cerebellum with extension across the vermis into the medial aspect of the right cerebellar hemisphere. (**a**) T1 post-gadolinium. (**b**) T2-weighed image

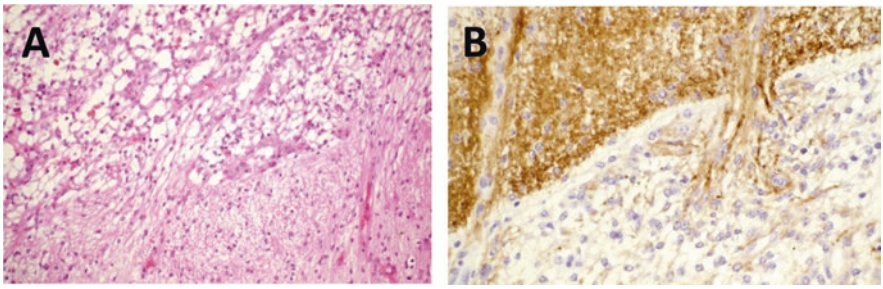

Fig. 4 (**a**) Pilocytic astrocytomas are characterized by a biphasic tumor architecture, with a solid fibrillary and a loose microcystic component (H&E). (**b**) Strong expression of the astrocytic marker glial fibrillary acidic protein (GFAP) is present in bipolar tumor cells of solid fibrillary areas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

assessment to treatment of pediatric low-grade gliomas, including PAs, have been published [34].

Tumor Pathology (Fig. 4)

Pilocytic astrocytomas (PAs), WHO grade I, are macroscopically soft, grey, 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 hair-like ("piloid") processes. Rosenthal fibers, amorphous sausage-like eosinophilic structures, 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 non-neoplastic lesions. Many PAs are rich in vasculature, most often hyalinized vessels are present but serpent-like 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, 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 [122]. Necrosis is often present but not associated with anaplasia. The prognosis of these "*anaplastic"* pilocytic astrocytomas is better than in glioblastomas. However, the most recent WHO CNS tumor classification does not formally recognize this entity; certainly, PAs with histologic features of anaplasia are more common in adults [84, 92].

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 [51], and approximately 50% harbor the most common BK fusion variant [57]. 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 the cerebellar location. PMA is characterized by monomorphous bipolar tumor cells, often in angiocentric arrangement, and myxoid tumor matrix [143]. 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 [81] and the entity is no longer provided with a distinct classification in the 2021 update [84].

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 has 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 [62, 92, 138]. Ryall et al. [126] integrated tumor molecular genetics in over 1000 clinically annotated pediatric low-grade gliomas and demonstrated that ~85% of these tumors harbored a driver genetic alteration of the RAS/MAPK pathway and that most of the remaining tumors in which a driver alteration could not be identified, there was evidence of RAS/MAPK pathway upregulation [126].

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%) [11, 13, 58, 62]. The N-terminal end of KIAA1549 replaces the N-terminal end of BRAF, producing a constitutively activated BRAF kinase domain and activation of the Ras/ERK pathway [13, 38, 64, 133]. 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 [63, 160]. 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 [26, 38, 63, 112, 160].

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 and can be identified in other pediatric and adult CNS tumors, including pleomorphic xanthoastrocytoma and ganglion cell tumors (formerly gangliglioma) [13, 65, 84, 128, 133]. The *BRAFinsT* mutation has also been described in PA in up to 3% of tumor samples specifically in the young adult population [29].

Multiple additional genetic alterations have been described in PAs. *KRAS* somatic mutations occur at low frequency (3–5%) [27, 42]. Aberrations affecting *FGFR1*, including point mutations (P.N546K, P.K656E), FGFR1-TACC1 fusions, and internal tandem duplications have been identified [63, 134, 160]. NTRK family receptor

kinase mutations have also been reported at a low rate, due to gene fusions leading to kinase activation [63, 160]. FGFR1 alterations are more frequent in midline structures, whereas BRAF V600E and NTRK family fusions are more frequent in supratentorial tumors [75, 132, 140]. There is unknown significance of reported 41% of PAs having MYB protein upregulation. Genomic alterations of MYB have only been found in diffuse gliomas [138] with the designation of a distinct classification in the WHO CNS 5 update of diffuse astrocytoma, MYB- or MYBL1 altered or angiocentric glioma [84].

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 [9, 27, 78, 79, 130]. In general, patients with NF1-associated PAs may have a more indolent course and are less likely to require treatment. If treated with carboplatin and vincristine chemotherapy, NF1 patients with PA have better EFS and experience less toxicity [6].

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 [59, 75]. Both transcriptome and methylome analyses revealed a distinctive pattern for infratentorial versus supratentorial PA [75, 132, 140].

Therapy and Prognosis

Overall, PA has an excellent prognosis with 10-year survival over 90% [18]. The treatment is primarily surgical and prognosis depends on the completeness of the resection. Patients who undergo sub-total resection are often treated with chemotherapy at tumor progression to improve long-term survival. Until recently, the standard of care has been carboplatin with vincristine, both administered intravenously (I.V.) [99, 101] and demonstrated less toxicity than an alternative multi-agent regimen in the COG A9952 randomized clinical trial [7]. Vinblastine given weekly I.V. has demonstrated similar efficacy as the carboplatin/vincristine regimen, with partially non-overlapping toxicities. Lassaletta et al., on behalf of the Canadian Pediatric Brain Tumour Consortium, assessed the activity of vinblastine in 54 therapy-naïve children with pediatric low-grade gliomas, of which almost half were PAs. Five-year OS and PFS were 94.4% and 53.2%, respectively [76]. Radiation therapy is effective but in clinical neuro-oncology practice it is often deferred in pediatric patients and young adults until two or more salvage therapies using either chemotherapy or targeted therapies have failed. 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 [113]. Infrequently major postoperative sequelae occur, such as postoperative posterior fossa mutism syndrome (<5% of patients) or marked new brainstem or cerebellar deficits [96]. More commonly, mild fine motor or balance issue occur, but often do 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, 162].

Current and Future Considerations

Although targeted therapies may eventually 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 [21]. Nobre et al. retrospectively reviewed 56 patients with pediatric low-grade gliomas; the majority were PAs. 80% of these patients demonstrated objective responses compared to less than 30% treated with conventional chemotherapy. While >75% of responding patients progressed after discontinuing BRAF inhibitors, 90% of this cohort responded upon BRAF inhibitor rechallenge [95]. In recently completed Phase I/II clinical trials using MEK inhibitors, such as selumetinib and trametinib, there was demonstrated efficacy in both NF1 and non-NF1 patients with PA and other pediatric low-grade gliomas along with a tolerable side-effect profile [10, 32, 33, 114].

Since resistance to BRAF inhibitors is often encountered, combination with a MEK inhibitor is under study in recurrent/progressive disease [37]. Bouffet and colleagues recently reported an interim analysis of a randomized Phase II study comparing first-line dabrafenib and trametinib versus carboplatin and vincristine in newly diagnosed patients with BRAF V600E mutations (NCT02684058). The ORR (CR + PR) and median PFS were 47% and 20.1 months with dabrafenib and trametinib versus 11% and 7.4 months with carboplatin plus vincristine, respectively. The safety profile was very good [15]. Future clinical trials for pediatric low-grade gliomas including PAs will incorporate molecular genetic tumor profiling and targeted therapies will be carefully integrated, including NTRK and FGFR inhibitors where indicated [92, 102, 137].

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 [82, 87]. 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 [139]. 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 [82]. 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 to 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 [22]. On T1-weighted MR imaging, ependymomas are usually hypointense or isotense 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 methemoglobin, hemosiderin, necrosis, calcification, encased native vessels, or tumor vascularity are commonly seen [22] (Fig. 5). It is important to image the entire craniospinal axis, as neuroaxis dissemination can occur in 3–11% of cases [111].

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.

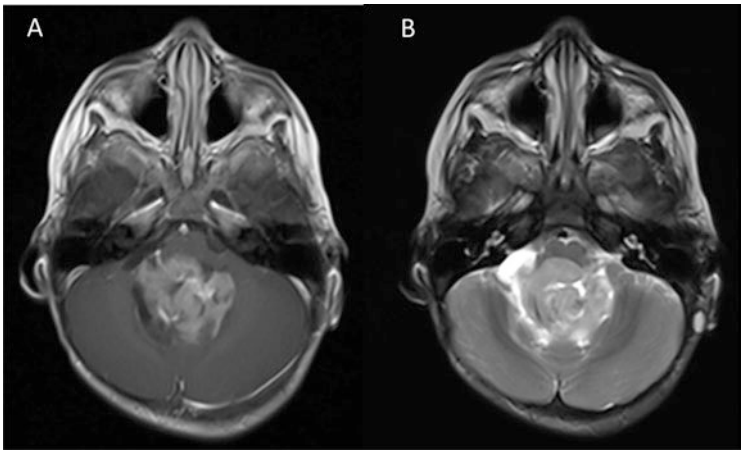

Fig. 5 15-month male with ependymoma with cystic and solid components localized to the fourth ventricle measuring 3.9 × 3.4 cm. (**a**) T1 post-gadolinium image. (**b**) T2-weighted image

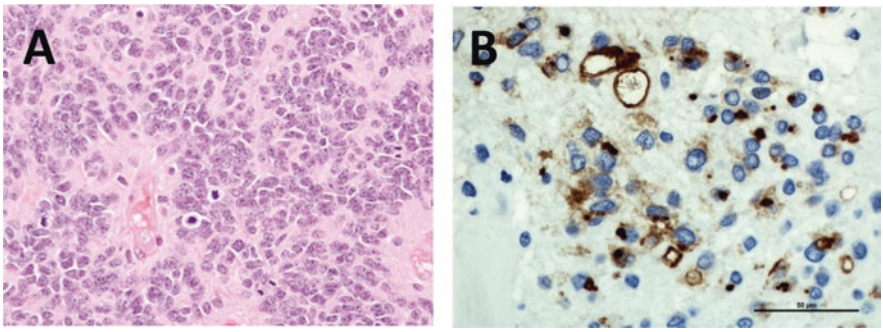

Fig. 6 (**a**) Ependymoma, WHO grade III, with markedly increased mitotic activity. Five mitotic figures are seen in this high-power field. (**b**) Cytoplasmic dot- and ring-like immunoreactivity for epithelial membrane antigen (EMA) in an ependymoma, grade III

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, 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. One interesting subgroup, the Trisomy 19 ependymomas, are WHO grade III, usually located in the supratentorial compartment and are often associated with chromosome 9 deletions and/or deletion of 13q21.31-31.2 [125]. In the WHO CNS 5 classification, ependymomas may be classed as either WHO grades II or III, but the term anaplastic ependymoma is no longer used [30, 84].

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 ring-like cytoplasmic immunopositivity for EMA (Fig. 6b). In contrast to supratentorial ependymomas, expression of L1CAM, which indicates rearrangement of *ZFTA* (zinc finger translocation associated), formerly known as *C11orf95* [84], is not detectable in posterior fossa ependymomas.

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 analyses have 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 *ZFTA* (*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 [108]. A second recurrent gene fusion product, *ZFTA* (*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 [104]. Three recent studies have further provided important insights into these ZFTA fused supratentorial ependymomas (*ZFTA*fus ST-EPN) [5, 73, 161].

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) ([155]; 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 [85, 104]. Furthermore,

Table 3 Posterior fossa ependymoma summary

	PFA	PFB
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	Ependymoma, WHO grade II/III Loss of H3 K27me3 expression	Ependymoma, WHO grade II/III H3 K27me3 retained

loss of chromosome 6q identifies a very high-risk group of PFA ependymomas [12]. Upregulation of multiple cancer-related signaling pathways has been observed, although they are not specific to ependymomas, including PDGFR, EGFR, VEGF, MAPK, and TGFβ [151, 155]. 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 (K27) on histone H3 and is responsible for silencing genes involved in cell differentiation and tumorigenesis [36, 97]. Of clinicopathological significance, there is frequently global loss of repressive Histone H3K27me3 (trimethylation) or overexpression of EZHIP (enhancer of Zeste homologs inhibitory protein, formerly *Cxorf67*) in PFA ependymomas, that can readily distinguish them from PFB tumors [56, 84, 105, 116]. Subsequent methylation array profiling of 675 PFA ependymomas identified 2 subgroups, PFA-1 and PFA-II, and 9 subtypes [103]. Panwalker et al. [106] also identified increased expression of H3 K27 acetylation (H3K27ac) in PFA tumors, including enrichment of this activating mark at several important glycolytic and TCA cycle-related genes, such as hexokinase-2 and pyruvate dehydrogenase [106].

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 prognosis [72, 155]. PFB ependymoma does not demonstrate the epigenetic modifications and hypermethylation profiles when compared to PFA tumors. However, insights into the molecular heterogeneity of PFB tumors provide some guidance toward future therapeutic strategies [20].

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 [151]. As a result of these key discoveries, novel therapeutic strategies can now be tested that target PRC2 and alter DNA methylation status in ependymoma [43, 127].

Therapy and Prognosis

Overall the prognosis for ependymoma is relatively unsatisfactory with overall survival reported as 50–71%. For posterior fossa ependymoma, molecular subgroup is an important prognostic factor independent of age and extent of resection [117]. 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 [90]. Survival of patients with GTR ranges from 66 to 80%, compared to sub-total 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 [88]. 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 3D conformal 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 [80]. However, chemotherapy has not made a significant impact on this disease [68]. The COG ACNS0121 clinical trial reinforced the backbone of maximal safe resection and adjuvant conformal RT to the involved field but did not demonstrate the efficacy of a chemotherapy window in children with ependymoma [91]. The SIOP Ependymoma I clinical trial reinforced the conclusions of ACNS0121. However, there was a modest response of some patients to VEC chemotherapy, with the chemotherapy response rate of 65.5% exceeding the pre-specified 45% [121].

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 [144, 159]. Reoperation, when safe to proceed, and re-irradiation (either to the involved field or craniospinal) is the usual treatment strategy for locally recurrent/progressive posterior fossa ependymoma [86, 146]. Many patients are offered oral or intravenous etoposide (CNS 2001 4, [4]), but the clinical impact has not been very encouraging.

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 or are ongoing, results have been less than promising [127]. Given the more common presentation of the

genomically "bland" PFA tumors in childhood, epigenetic, metabolic (e.g., metformin) and CAR T-cell directed therapies (including HER2- and B7-H3-specific) may hold more promise [68, 106, 127, 157].

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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 pediatric brain tumor. Recent advances in sequencing technologies have revolutionized molecular classification of these highly aggressive tumors. MB is divided into 4

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molecular subgroups that exhibit different genomic alterations, gene expression profiles, response to treatment, and developmental cell of origin: WNT, Sonic Hedgehog (SHH), Group 3, and Group 4. Additional stratification into as many as 14 molecular subtypes underscores the extensive heterogeneity and complexity both between and within the major subgroups. While targeted therapies are being evaluated, the current treatment for MB still consists of aggressive surgery, high doses of cytotoxic chemotherapy, and radiation to the whole brain and spinal cord. These treatments do not take into account the extensive heterogeneity between and within MB subgroups. 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 including proton-based radiotherapy, histological classifications, and a detailed description of the current molecular subgroups and subtypes, followed by exploration of cellular heterogeneity in the molecular era. Further dissection of tumor heterogeneity and identification of subgroup and 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)

Medulloblastoma (MB) is the most common primary malignant pediatric brain cancer in North America [1]. Primary MB tumors typically develop in the cerebellum and fourth ventricle with frequent dissemination through the cerebrospinal fluid (CSF) driving metastasis and tumor recurrence [1]. MB is 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. 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 cerebello-pontine 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. Hoarseness and swallowing difficulties are less common but may be present at the time of diagnosis due to lower cranial nerve dysfunction.

(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 may be present such as facial weakness, hoarseness, or swallowing difficulties.

(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*

Up to 30% of patients with MB will have disseminated disease at the time of diagnosis; however, 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 current treatment for MB consists of aggressive surgery followed by high doses of cytotoxic chemotherapy and radiation to the whole brain and spinal cord 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 (or average) risk disease with 80–85% overall survival. These patients have total or near total resection of their tumors and no evidence of dissemination in the CSF [6, 7]. Michalski et al. recently [8] described the results of the COG ACNS0331 (NCT00085735) randomized clinical trial for patients with average and low-risk disease. Of significance, a radiation boost to the involved field was not inferior to a boost to the entire posterior fossa, the former standard of care (hazard ratio 0.97). However, the overall dose reduction of craniospinal irradiation (CSI) from 24.3 Gy (standard dose) to 18 Gy in children aged 3–7 years was inferior with a hazard ratio of 1.67.

The second treatment group consists of children greater than 3 years of age with high-risk disease. This is defined as the presence of greater than 1.5 cm^2 of residual tumor after surgery or dissemination/metastasis with large cell/anaplastic histology. In the recently published results of the COG ACNS0332 (NCT00392327), Leary et al. [9] described the outcomes for 261 evaluable patients, 72% with metastatic disease. Five-year event-free survival (EFS) was 66.4% and overall survival (OS) was 73%. The 13-cis-retinoic acid maintenance arm was closed early due to clinical futility. Of interest, in patients who received weekly intravenous carboplatin during CSI, 5-year EFS was 66.4% compared with 59.2% in patients not receiving carboplatin. Yet, with molecular subgroup analysis (see below), patients with Group 3 MBs had a 73.2% 5-year EFS with carboplatin compared to 53.7% without, accounting for almost all the patients for whom carboplatin provided improved outcomes. 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 (infants) constitute a separate treatment group. Although radiation therapy consisting of CSI with a boost to either the posterior fossa or involved field 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 at diagnosis. These patients are treated with high-dose chemotherapy to delay or remove the need for radiation therapy and allow the nervous system an opportunity to further develop [10].

Proton Versus Photon Radiotherapy – The Emerging Standard of Care for MB

In the past decade, there has been a paradigm shift in how radiation therapy (RT) is delivered to children, especially those with brain tumors including MB. Tumor-targeting using protons, a positively charged molecule with mass, results in less radiation to surrounding anatomic structures in the brain and body, thereby having the potential to reduce long-term neurocognitive and neuroendocrine sequelae in survivors. However, using protons increases LET (linear energy transfer) and RBE (relative biological effectiveness), with the potential for increased neurotoxicity,

such as brainstem injury, radiation necrosis, and the development of second malignant neoplasms (SMN). Although photon-based linear accelerators remain the international standard of care, in the USA, UK, Europe, and Japan, proton beam therapy (PBT) units have been established with patterns of referral from countries, such as Canada and Australia, where this therapy is being implemented or planned.

In 2016, Yock et al. [11] showed a global decrease in IQ points at 1.5 points/year with protons. In total, 55% of patients experienced neuroendocrine deficits, including growth hormone (GH) deficiency. There was only one late brainstem injury. In another study involving a mixed cohort of children with brain tumors, Kahalley et al. [12] established that photon-based RT results in an overall reduction of 8.7 global IQ points compared to protons ($p = 0.011$). A landmark follow-up study by the same group [13] focused exclusively on MB demonstrated superior neurocognitive outcomes in children and adolescents who received proton-based RT, including global IQ, perceptual reasoning, and working memory. However, there was no difference between photons and protons for verbal reasoning or processing speed.

In a cohort of 88 patients with standard risk MB, patterns of failure following protons and photons were similar [14]. Giantsoudi et al. [15] studied a cohort of 111 patients and identified only 3.6% with any evidence of CNS injury; there was an increased risk in patients who received a radiation boost to the posterior fossa rather than the involved field. Eaton et al. [16] evaluated 77 children with standard risk MB. Compared to those who received photon-based RT, patients treated with protons had decreased hypothyroidism (23% versus 69%), sex hormone deficiency (3% versus 19%), and fewer required endocrine replacement therapy (55% versus 78%). However, there were no significant differences regarding GH deficiency or the incidence of precocious puberty.

Baligaet al. [17] established the 10-year cumulative incidence of SMN at 5.6% and brainstem injury at 2.1% using proton-based RT. In another study focused on the incidence of second tumors, Indelicato et al. [18] evaluated their large referral-based proton RT database ($n = 1713$; 22% were less than 3 years of age). Of this population, 2.2% had a tumor predisposition syndrome such as type 1 neurofibromatosis or Li Fraumeni Syndrome. However, only 11 patients developed an SMN with a calculated 5- and 10-year cumulative incidence of 0.8% and 3.1%, respectively; 10/11 received proton RT at less than 5 years of age.

Histological Classification

Traditionally, MB has been classified based on histological properties. MB appears as a small round blue cell tumor. This is a characteristic seen upon hematoxylin and eosin (H&E) staining attributed to the presence of large nuclei and scant cytoplasm in less differentiated cells [19]. MB can be divided into four main histological variants. They are known as classic, large cell/anaplastic (LCA), desmoplastic/nodular, and MB with extensive nodularity (Fig. 1) [20].

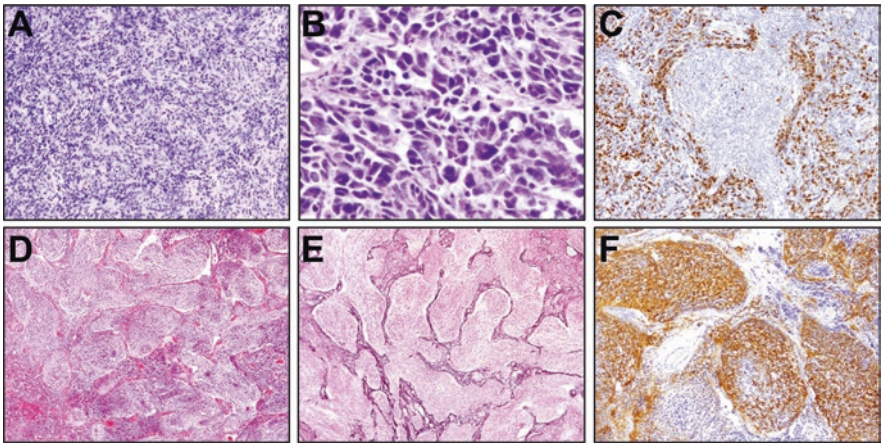

Fig. 1 (**a**) Classic medulloblastoma (MB), WHO grade IV, hematoxylin and eosin (H&E). (**b**) Large cell/anaplastic MB, WHO grade IV (H&E). (**c**) Desmoplastic/nodular MB, WHO grade IV stained with Ki67, a proliferation marker. (**d**) MB with extensive nodularity, WHO grade IV (H&E). (**e**) MB with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

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 which are circular nuclear arrays with fine tangled cytoplasmic processes [20]. 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 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 [21]. Nuclear accumulation of β-catenin is associated with excellent clinical outcomes in these tumors [21, 22].

Large Cell/Anaplastic (LCA) MB is the most malignant histologic variant and is characterized by nuclear pleiomorphism with large nuclei, prominent nucleoli, and abundant cytoplasm (Fig. 1b) [20, 23]. 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, while 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 [20, 23, 24]. The nuclear: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 [20]. Immunohistochemically, Ki67 labeling index is higher in internodular zones reflecting the higher mitotic activity of the typically undifferentiated cells present in these areas [20]. 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 [25] (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 MB 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 reliably classify MB tumors while better predicting clinical outcome and enabling more appropriate selection of treatment options. The advancement of multi-omic sequencing technologies has led to the complete restructuring of the different types of MB and this has now been incorporated into the World Health Organization (WHO) Classification of Central Nervous System Tumors [1].

The first gene expression array profiling studies [26–29] led to the stratification of MB into 4 consensus molecular subgroups: WNT, Sonic Hedgehog (SHH), Group 3, and Group 4 (Fig. 2) [19, 26–29]. These initial findings were followed by large-scale next-generation sequencing (NGS) studies of primary MB samples that provided deep insight into their heterogeneity and biological complexity [30–32]. Indeed, the 4 distinct molecular subgroups exhibit different genomic alterations, gene expression profiles, and response to treatment (Fig. 2). Following the

integration of this new molecular classification scheme by the WHO in 2016, deeper analyses of clinical trends and molecular features further resolved each of the 4 major subgroups into multiple subtypes [33–35], with the most recent consensus outlined below for each. No longer considered a single disease entity, these studies underscored the highly heterogeneous nature of MB. The extensive multi-omic bulk tumor analyses on MB patient samples have recently been summarized in the comprehensive review by Hovestadt et al. [36].

This novel molecular classification system has been adopted by the WHO [1], can reliably predict patient prognosis, and has the potential to drive subgroup or even subtype-specific treatment regimens. Importantly, molecular subgrouping/subtyping has improved risk stratification, thus providing opportunities to reduce therapy for lower-risk groups like WNT and intensify therapy for the very high-risk Group 3 MB patients [37]. Sequencing has also revealed mechanisms of resistance to targeted therapies. For example, SHH pathway inhibitors are not predicted to work on tumors harboring mutations in downstream SHH pathway genes such as *SUFU* or *MYCN* [38–41]. While targeted therapies are currently being evaluated in clinical trials (SJDAWN (NCT03434262), SJELIOT (NCT04023669), or SJMB012 (NCT01878617)), over 30% of MB patients die while survivors are left with the long-term physical and cognitive side effects associated with chemotherapy and radiation [6]. For the highest risk groups of childhood MB patients, novel therapies are urgently needed.

The molecular subgroups differ in their demographics, gene expression profiles, somatic genetic events, clinical outcomes, and histology (Fig. 2) [1, 19]. The SHH and WNT variants are aptly named for the well-established signaling pathways that

Subgroup	WNT	SHH	Group 3	Group 4
Frequency	10%	30%	25%	35%
Peak age (years old)	3-10 and 10-17	0-3 and 17+	3-10	3-10
male to female ratio	1:1	2:1	2:1	2:1
Molecular and genetic alterations	WNT signaling *CTNNB1* mutation Monosomy 6	SHH signaling *PTCH1/SMO/SUFU* mutation *MYCN* amplification	Photoreceptor/GABAergic signaling TGF-β signaling *MYC* amplification	Neuronal/Glutamatergic signaling NF-κB signaling *CDK6* amplification Isochromosome 17q
Metastasis	Rare	Uncommon	Very frequent	Frequent
Prognosis	Good	Intermediate	Poor	Intermediate
Putative cell of origin	Mossy fiber neuron lineage from lower rhombic lip	Granule neuron progenitor cells (GNPCs) of the EGL	Stem/progenitor cell from the developing cerebellum (rhombic lip ventricular zone (RL^{VZ}) and rhombic lip subventricular zone (RL^{SVZ}))	Progenitor cell from the developing cerebellum (rhombic lip subventricular zone (RL^{SVZ}))

Fig. 2 MB is divided into 4 major molecular subgroups that exhibit different genomic alterations, gene expression profiles, response to treatment and developmental cell of origin. The 4 subgroups are WNT, Sonic Hedgehog (SHH), Group 3, and Group 4

drive tumorigenesis. Less is known about the molecular basis of disease progression for the most aggressive Group 3 MB tumors that exhibit the worst prognosis, as well as for Group 4 MBs. Each MB subgroup and their corresponding substructure or subtypes will now be discussed in detail below. Demographics, genetic/molecular alterations, cell of origin, mouse models, and treatment options for each will be described.

WNT-Activated MB

WNT tumors are characterized by the upregulation of genes associated with the WNT signaling pathway and have a favorable prognosis with a 5-year survival rate of 95% or better (Figs. 2 and 3) [19, 36]. The small percentage that do not survive long-term often succumb to complications from therapy or secondary neoplasms caused by radiation therapy rather than WNT MB recurrence [42].

Demographics

WNT tumors represent the smallest group of MBs at just 10% of diagnoses. These tumors have a nearly equal distribution between males and females [19]. WNT MBs occur primarily in children from 4 years of age to early adulthood with a peak incidence at 10–12 years of age and are not typically seen in infants [36]. Recently, the WNT subgroup has been further subdivided into two molecular subtypes (Fig. 3), WNT-α and WNT-β which differ in the age at diagnosis [33]. WNT-α tumors have a median age of 10 years old and WNT-β tumors have a median age of 20 years old [33]. WNT tumors are typically located along the midline of the fourth ventricle and infiltrate the brain stem. These tumors are usually of classic histology and infrequently metastasize [43].

Genetic and Molecular Alterations

The WNT signaling pathway (Fig. 4) plays a critical role in defining the midbrain-hindbrain boundary during brain development [44, 45] and is important for regulating self-renewal of neural precursors during neurogenesis [46]. The first indication that mutations in the WNT pathway caused a form of MB came from the study of patients with Turcot syndrome which is 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 [47, 48]. ß-catenin (encoded by *CTNNB1*) is the main signaling molecule in the canonical WNT signaling pathway [49, 50]. In the absence of WNT ligands, β-catenin levels are kept low in the cytoplasm.

Subgroup		WNT	
Subtype		WNTα	WNTβ
Demographics	Frequency (%)	70	30
	Age (years old)	Median age of 10	Median age of 20
	Gender (% male : % female)	45% male : 55% female	
Clinical features	Histology	Classic	
	Metastases	8.6%	21.4%
	Survival (at 5 years)	97%	100%
Molecular features	Driver events Cytogenetics	- *CTNNB1*, *DDX3X* or *SMARCA4* mutations - Monosomy 6	

Fig. 3 The Wingless (WNT) subgroup is divided into 2 different subtypes, WNTα and WNTβ, based on demographics, clinical, and molecular features. Adapted from Hovestadt et al. Nature Reviews Cancer, 2019 and Cavalli et al., Cancer Cell, 2017

ß-catenin levels are regulated by a multi-protein destruction complex [50]. 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 (GSK-3β) (Fig. 4) [50], leading to its eventual proteasomal destruction and subsequent gene target repression [49, 50]. 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 [51, 52]. However, in the presence of WNT ligands binding to frizzled (FRD) and its co-receptor LDL-receptor-related protein 5/6 (LRP), the proteasomal degradation of β-catenin is blocked resulting in an accumulation of stable β-catenin in the cytoplasm [53, 54]. In the nucleus, β-catenin displaces Groucho and activates TCF/LEF which increases the transcription of target genes such as cyclin D1 [55, 56] and the transcription factor MYC-proto-oncogene (*MYC*) [57, 58].

Somatic mutations in downstream WNT signaling pathway components such as *CTNNB1*, *AXIN1*, and *APC* are the hallmark genetic events defining this subgroup and are found in sporadic MB [59–63]. The majority of WNT subgroup tumors show stabilizing mutations in *CTNNB1* (70–90%) and monosomy 6 (90%) [19, 31, 64–66]. However, a small number of WNT MB tumors lack mutations in *CTNNB1* and *APC* implying that other mechanisms lead to aberrant WNT signaling and tumorigenesis in these cancers [64, 65]. WNT tumors lacking *CTNNB1* mutations have been reported to exhibit mutations in the cadherin-1 (*CDH1*) gene [64]. This

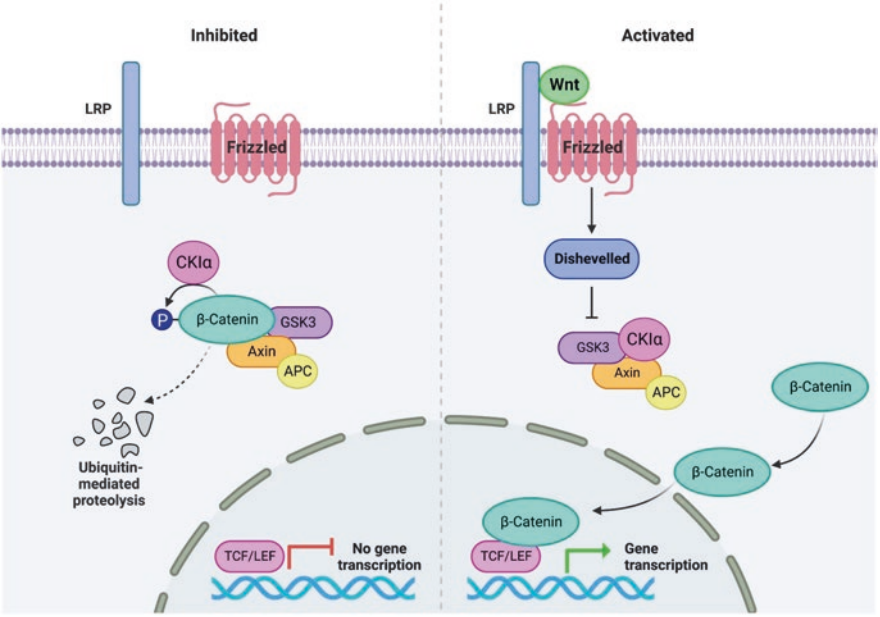

Fig. 4 The Wingless (WNT) pathway plays a critical role in WNT-activated MB tumorigenesis. Canonical (β-catenin dependent) WNT signaling plays a key role in the formation of the midbrain/hindbrain boundary through the control of neural stem cell proliferation and is believed to drive tumor initiation 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) leading to target gene 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. Adapted from "Wnt Signaling Pathway, Activation and Inhibition", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates

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 [67]. In addition to WNT signaling aberrations, 36–50% of WNT tumors show mutations in the DEAD Box Helicase 3 (*DDX3X*) gene [30–32, 64]. DDX3X is an RNA helicase that has been implicated in mRNA splicing and processing, translational control, chromosome segregation, cell cycle regulation, and cancer progression [68, 69]. Approximately 21% of SHH, but no Group 3 or 4 tumors, exhibit mutations in the *DDX3X* gene [31, 64]. Indeed, elegant work by Patmore et al. [70] has shown that *Ddx3x* is a Wnt- and Shh- MB tumor suppressor and key regulator of both normal and malignant hindbrain development. Ddx3x restricts cell lineage competence to form these MB subgroups, whereas *Ddx3x* deletion removed this inhibition enabling tumor formation [70]. Other mutations in genes such as SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 4 (*SMARCA4*), CREB-binding protein

(*CREBBP*), transformation/transcription domain-associated protein (*TRRAP*), and mediator complex subunit 13 (*MED13*), which are all regulators of gene expression through chromatin remodeling, have also been discovered in WNT tumors [64, 71–74]. Aberrant WNT signaling is the dominant molecular driver of this MB subgroup; however, in addition to stabilization of CTNNB1, formation of WNT MBs may require disruption of chromatin remodeling at WNT-responsive genes.

Cell of Origin and Mouse Models of WNT-Activated MB

Tumors of the WNT subgroup have been shown to arise from lower rhombic lip progenitor cells of the dorsal brainstem [75]. An activating mutation in *Ctnnb1* disrupts the normal differentiation and migration of these progenitor cells resulting in abnormal accumulation of cells [75]. Regional expression of 24 WNT-MB signature genes was charted using software that generates three-dimensional gene expression maps across the developing mouse brain [75]. 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 [75]. Based on these 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 brain lipid-binding protein (*Blbp*) gene, which includes the ventricular zone progenitors. *Ctnnb1* mutations were also expressed in granule neural precursor cells (GNPCs) using the enhancer of the atonal BHLH transcription factor 1 (*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 [75], 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 MBs that were confined to the dorsal brainstem and displayed expression profiles similar to WNT-subgroup MB [75]. 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 subgroup. Indeed, more recent single-cell RNA sequencing (scRNA-seq) studies by Jessa et al. [76] provided further support for these findings in mouse models by demonstrating that WNT MBs match to the lower rhombic lip pontine mossy fiber lineage in the brainstem.

Treatment

Patients with WNT MB undergo standard current treatment including surgery, chemotherapy, and radiation therapy. As WNT tumors exhibit a relatively good prognosis, it has been recommended that patients with non-metastatic disease receive reduced chemotherapy and radiation therapy [32]. While some are advocating to

remove the use of craniospinal irradiation (CSI) in patients with average risk WNT MB, COG ACNS 1422 (NCT02724579) offers reduced dose CSI to 18 Gy, omission of weekly vincristine during CSI and reduced doses of adjuvant lomustine and cisplatin chemotherapy. In fact, recent findings in a cohort of 93 WNT MB suggest that these studies should proceed with caution as maintenance chemotherapy was a strong predictor of relapse while those individuals treated with high-dose chemotherapy exhibited significantly improved outcomes [77]. WNT MBs have also been shown to exhibit a highly aberrant and leaky vasculature, enabling substantial accumulation of intra-tumoral chemotherapy [78] and likely contributing to the more robust clinical response. Clearly, more research is required to better understand the clinical behavior of this MB subgroup. Although there are small-molecule inhibitors of the WNT signaling pathway, crosstalk between signaling pathways necessitates treatment options that can target multiple pathways [79]. 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. [80] have shown that small molecule PI3K/AKT inhibitors such as OSU03012 decrease WNT signaling by activating GSK-3β and promoting degradation of β-catenin. The anti-cancer compound Norcantharidin (NCTD) has also been shown to impair nuclear translocation of β-catenin signaling and reduce tumor growth in an orthotopic mouse xenograft model of MB [81].

SHH-Activated MB

Demographics

The SHH subgroup is characterized by the upregulation of genes associated with the SHH signaling pathway. Mutations in the SHH signaling pathway represent the most common genetic events, including inactivating germline or somatic mutations and deletions of patched 1 (*PTCH1*) and of suppressor of fused homolog (*SUFU*) as well as activating mutations in smoothened homolog (*SMO*) and amplifications of glioma-associated oncogene family zinc finger 2 (*GLI2*) [35, 41]. Demographically, SHH MB is more common in males than in females with approximately a 2:1 ratio (Fig. 2). SHH MB displays a bimodal age distribution with most cases occurring in both infants (<3 years of age) and adults (>17 years of age) and fewer cases being diagnosed during childhood and adolescence [19, 26]. These tumors make up approximately 28% of all MBs diagnosed and have an intermediate prognosis [19]. SHH MBs frequently arise in a cerebellar hemisphere in adults [75] and in the cerebellar vermis in children [26].

The 5-year overall survival (OS) rate in infants is 77%, which drops to 68% in children and 75% in adults [66]. This difference in survival between age groups is most likely attributed to the high percent of infant SHH tumors exhibiting desmoplastic/extensive nodularity which has been shown to be a positive prognostic factor

in these young patients [82]. The majority of all SHH tumors are described as either having classic or desmoplastic/nodular histology with the remainder being large cell/anaplastic (LCA) histology. Nearly all desmoplastic/nodular variants are of the SHH subtype; however, 50% of all SHH tumors are not desmoplastic [19]. This highlights the importance of incorporating additional molecular profiling into diagnostics. Moxon-Emre et al. [83] investigated the intellectual outcomes of 121 patients treated with MB from 1991 to 2013. Of interest, those with an SHH tumor had a distinct neurocognitive phenotype, with less decline in processing speed. Moreover, this molecular subgroup had the lowest incidence of cerebellar mutism (also referred to as posterior fossa syndrome), which occurs in up to 25% of MB patients post-operatively [84–86].

Genetic and Molecular Alterations

The SHH signaling pathway (Fig. 5) 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 [87–89] and is believed to drive tumor initiation in the SHH MB subgroup [19]. The membrane-bound receptor PTCH plays an inhibitory role that represses SHH signaling when it is unbound [90]. Binding of the SHH ligand to PTCH releases the inhibitory effect PTCH has on SMO, a member of the G protein-coupled receptor family [91]. De-inhibition of SMO results in the activation of the zinc-finger proteins of the GLI transcription factor family including GLI1, GLI2, and GLI3 [90]. 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 (GLIr) forms [90]. With the activation of SMO, transcriptionally active forms of GLI (GLIa) are formed in combination with inhibition of suppressor of fused (SUFU), a protein responsible for sequestering GLI in the cytoplasm (Fig. 5) [90, 92]. 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 [92].

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 [19, 93]. 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 subgroup. 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 subgroup [26, 30, 94]. Other genomic abnormalities include gain of chromosomes 2 and 9p, and loss of 10q, 14q, and 17p [30, 95]. For a thorough summary of

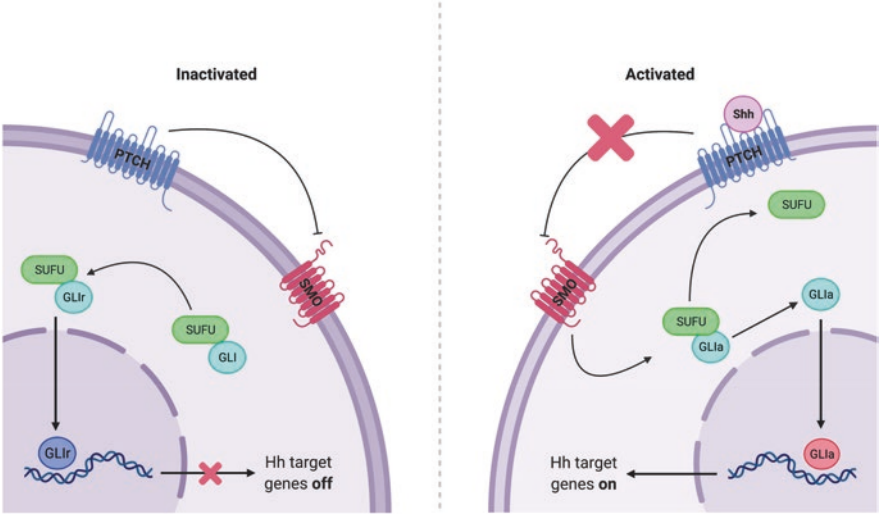

Fig. 5 The Sonic Hedgehog (SHH) signaling pathway plays a critical role in SHH-activated MB tumorigenesis. The SHH signaling pathway plays an essential role in the control of GNPC proliferation in the EGL and is believed to drive tumor initiation in SHH MB. When SHH is not present, the Patched (PTCH) 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 activated GLI to the nucleus. Adapted from "Hedgehog Signaling Pathway", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates

the genomic alterations associated with SHH MB, see the recent excellent review by Garcia-Lopez et al. [96].

The extensive heterogeneity observed at the genetic and molecular levels has led to the discovery of additional subtypes within SHH MBs [97]. 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 [97]. In addition, 72% of patients aged 5 or older who succumbed to their disease had harbored a *TP53* mutation. *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 [97]. *TP53*-wildtype patients have a 5-year OS of 81% whereas patients with *TP53*-mutations exhibit a 41% 5-year OS [97].

More recent studies by Cavalli et al. [33] have provided even further insight into the heterogeneity within SHH MBs by subdividing this subgroup into 4 distinct subtypes: SHHα, SHHβ, SHHγ, and SHHδ (Fig. 6). SHHβ and SHHγ correspond to infant subtypes, whereas SHHα and SHHδ correspond to childhood/adolescent and adult subtypes, respectively [33]. Adult SHH MBs are characterized by a higher

prevalence of SHH pathway-associated mutations (including higher incidence of *PTCH1* and *SMO* alterations) and a more expansive list of chromatin modifier mutations [41]. Virtually all such MBs also harbor telomerase reverse transcriptase (*TERT*) promoter mutations [98, 99]. Interestingly, a recent study also identified highly recurrent mutations in the non-coding U1 spliceosomal small nuclear RNA (U1 snRNA) in 97% of adult SHH MB (SHHδ) [100]. These mutations lead to *PTCH* inactivation and *GLI2* and *CCND2* activation, opening up the intriguing possibility of targeting the spliceosome in SHH MB tumors [100]. Subtype SHHα is enriched for *GLI2* and *MYCN* amplifications as well as *TP53* mutations which have been shown to confer a poor prognosis [97], compared with patients with the SHHδ subtype which have a more favorable prognosis. SHHβ consists of infants aged 0–3 years old and has a lower 5-year survival rate compared to the SHHγ subtype which also consists of infants but with a better prognosis [33]. Of note, the most recent WHO classification of Central Nervous System Tumors has redesignated the SHH MB subtypes as SHH-1 (−β), SHH-2 (−γ), SHH-3 (−α), and SHH-4 (−δ) (Fig. 6) [1].

Cell of Origin and Mouse Models of SHH-Activated MB

During normal cerebellar development, 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

Subgroup		SHH			
Subtype		**SHHα** (SHH-3*)	**SHHβ** (SHH-1*)	**SHHγ** (SHH-2*)	**SHHδ** (SHH-4*)
Demographics	Frequency (%)	29	16	21	34
	Age (years old)	>3-10, >10-17	0-3	0-3	17+
	Gender (% male : % female)	63% : 37%	47% : 53%	55% : 45%	69% : 31%
Clinical features	Histology	Classic > Desmoplastic > LCA	Desmoplastic > classic	Desmoplastic > MBEN classic	Classic > desmoplastic
	Metastases	20%	33%	8.9%	9.4%
	Survival (at 5 years)	70%	67%	88%	89%
Molecular features	Driver events	- *MYCN* or *GLI2* amplification - *TP53* mutation - *PTCH1* mutation	- *PTCH1* mutation - *SUFU* mutation/ deletion - *PTEN* deletion	- *PTCH1* or *SMO* mutation - *PTEN* deletion	- *PTCH1* mutation - *TERT* promoter mutation

Fig. 6 The Sonic Hedgehog (SHH) MB subgroup is divided into 4 different subtypes based on demographics, clinical features, and molecular features: SHHα, SHHβ, SHHγ, and SHHδ. These have since been redesignated as SHH-1 (−β), SHH-2 (−γ), SHH-3 (−α), and SHH-4 (−δ) in the 2021 WHO Classification of Central Nervous System Tumors. Adapted from Hovestadt et al. Nature Reviews Cancer, 2019 and Cavalli et al., Cancer Cell, 2017.* New designations based on WHO Classification of Central Nervous System Tumors, 2021

anatomical region where SHH tumors originate. Schüller et al. [101] 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. [102] showed that deletion of *Ptch* and over-activation 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 [101, 102]. Recently, Zhang et al. have shown that OLIG2+ progenitor cells can drive SHH subgroup MB tumorigenesis in mice and are enriched in recurrent or resistant SHH MB [103]. They used single-cell transcriptomic analyses to demonstrate a developmental hierarchy of progenitor pools in SHH MB and identified OLIG2-expressing glial progenitors as transit-amplifying cells at the tumorigenic onset [103]. OLIG2+ progenitor cells are quiescent stem-like cells in full-blown MB but re-emerge during relapse and are highly enriched in therapy-resistant and recurrent SHH MB. These results demonstrate that OLIG2+ glial progenitor cells are critical tumor-initiating cells during MB tumorigenesis and relapse that could have important implications for the design of therapies to target cell lineage vulnerability during MB tumorigenesis and recurrence [103].

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 [104–106]. Mouse xenograft models are also used where cultured human SHH MB cells are injected into the cerebellum of immunodeficient mice. Patient-derived xenograft (PDX) lines are generated by implanting patient cells directly into the cerebellum of immunodeficient NSG mice and propagating them from mouse to mouse without in vitro passaging. PDXs are considered to be "gold standard" in vivo models. However, recent studies have shown differences in blood-brain-tumor barrier (BBTB) integrity between PDX and genetically engineered mouse models. This is important and should be considered in the design of preclinical studies to test novel therapeutics [107].

Treatment

Activation of the SHH signaling pathway in SHH MBs has been extensively studied. Accordingly, SMO inhibitors such as cyclopamine and vismodegib have been evaluated for MB patients with SHH pathway activation, especially in the setting of recurrent disease [108]. Cyclopamine is a naturally occurring small molecule inhibitor that suppresses SHH signaling by binding to SMO [109, 110]. However, the

potency of cyclopamine is relatively low and therefore synthetic SMO inhibitors such as vismodegib, sonidegib, and saridegib have also been utilized [111–114]. SMO inhibitors are initially successful, but 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 [41, 108, 115]. Furthermore, patients with *TP53* mutations are resistant to vismodegib treatment [97]. 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 [116]. Whole-genome sequencing in human samples also demonstrated genetic divergence between matched tumors at diagnosis and post-therapy [116]. Thus, targeted therapy against the primary tumor will most likely be ineffective against recurrent disease resulting in failed clinical trials.

As SHH tumors also exhibit upregulation of other signaling pathways, crosstalk between pathways may play a role in treatment resistance. There is conflicting evidence regarding the importance of the NOTCH pathway in SHH-activated MBs. Hallahan et al. demonstrated that targeting the NOTCH pathway with ϒ-secretase inhibitors decreases proliferation and increases apoptosis in an SHH MB xenograft model [104]. However, Hatton et al. have shown that targeting the NOTCH pathway is not beneficial in SHH MBs [117]. Liang et al. have recently identified novel roles for the CD271/p75 neurotrophin receptor and the MEK/ERK signaling pathway in contributing to SHH MB growth and tumor progression [118]. Bioinformatics analyses of large patient datasets and tumorspheres from SHH MB cultures demonstrated that CD271 is a novel and promising diagnostic marker for these tumors [118, 119]. CD271+ cells exhibit upregulated MEK/ERK signaling and inhibiting this pathway reduced endogenous CD271 levels, stem cell proliferation, survival, and migration in vitro [118]. The MEK inhibitor selumetinib crosses the blood-brain barrier and has been extensively tested in clinical trials for the treatment of other pediatric cancers like low-grade glioma and plexiform neurofibromas associated with type 1 neurofibromatosis [120–122]. Interestingly, treatment with selumetinib extends survival and decreases CD271 levels in vivo providing the first evidence that the MEK/ERK pathway is a therapeutic target in human SHH MB [118]. The MAPK signaling pathway has also recently been shown to drive SHH pathway inhibitor resistance [123]. Zhao et al. demonstrated that MAPK pathway activation is increased in metastatic SHH MB [123]. While Liang et al. show that selumetinib treatment significantly extends survival in an intracerebellar transplant model of SHH MB, the mice still succumb to disease progression [118]. The combination of selumetinib with the JAK/STAT3 pathway inhibitor pacritinib has recently been shown to further reduce tumor growth and increase survival in preclinical mouse xenograft models [124]. However, future work will continue to focus on identifying therapeutics that act synergistically or in combination with selumetinib to further attenuate tumor growth. Several studies have shown that genes associated with PI3K pathway contribute to SHH MB progression and drug resistance in addition to the MEK/ERK pathway [39, 125, 126]. PI3K pathway inhibitors in combination with SHH pathway inhibitors have demonstrated enhanced efficacy and improved survival in MB orthotopic xenograft mouse models [39,

127]. This provides further evidence that a combinatorial treatment approach will be necessary to treat SHH tumors. Additionally, a major hurdle in the development of effective therapies for MB is the impaired delivery of systemic therapies to tumor cells due to a specialized endothelial blood-brain barrier. Drug delivery across the BBTB is critical for the successful translation of novel therapies to treat brain tumors. Genovesi et al. [107] showed that BBTB integrity is highly variable in preclinical models of MB. This raises questions as to the scope of the translational relevance of these models. The authors highlight the importance of characterizing the functional status of the BBTB in preclinical models of MB and propose that these methods should be adopted more broadly in preclinical drug discovery studies for pediatric brain tumors [107].

Group 3 and Group 4 MB

These two "non-SHH/WNT" subgroups share similar clinical presentations and molecular characteristics and will therefore be discussed together.

Demographics

Group 3 MB occurs predominantly in infants and children and is rarely seen in patients older than 18 years of age, whereas Group 4 MB occurs across all age groups [19] (Fig. 2). Group 3 makes up approximately 25–30% of MB diagnoses and Group 4 is the most common MB subgroup with a frequency of 35% [94]. However, Group 4 is the least understood [19]. The majority of Group 3 and Group 4 tumors present with classic histology, with some desmoplastic and LCA cases [19]. LCA histology is more prevalent in Group 3 than in Group 4 tumors. Group 3 patients have the worst prognosis of the four subgroups with infants having a 5-year OS of 45% and children having a 5-year OS of 58% [94]. Group 3 MB tumors have a very high rate of metastasis which is a major contributor to their poor prognosis. Group 4 MB tumors have an intermediate prognosis, similar to the SHH subgroup [19]. Both Group 3 and Group 4 MBs occur in a 2:1 ratio in males compared to females and 30–40% of patients are metastatic at diagnosis in both subgroups [29]. Early nomenclature did not always separate Group 3 and Group 4 into distinct subgroups and in some cases described them as a single "mixed" subgroup of patients designated as "non-WNT/non-SHH" MB because they share similar clinical presentations and molecular characteristics [29]. To reconcile this issue, the two subgroups have recently been subdivided into 8 different subtypes: I, II, III, IV, V, VI, VII, and VIII (Fig. 7) [35, 36].

Subtype I is the least common while Subtypes II and III are associated with poor survival, and Subtype IV tumors have a more favorable outcome in non-infant patients. Group 4 MBs mostly make up Subtypes V, VI, and VII but these subtypes

also include some Group 3 tumors [35, 36]. Subtype VIII is purely Group 4, mostly occurs in older children and is the most common. While Subtype VIII is associated with favorable 5-year survival; however, many patients with this subtype are affected by late relapse and death [35, 36]. Overall, the extensive variation both between and within subgroups highlights the power of the molecular subtype classification system.

Genetic and Molecular Alterations

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 [26, 28, 31]. Other cytogenetic changes seen in Group 3 and 4 tumors include: 17p deletion, gain of chromosome 1q, and loss of chromosome 5q and 10q. Group 3 tumors are more likely to show gain of chromosome 1q and/or loss of chromosome 5q and 10q [19]. 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 [31, 64, 71–74]. Mutations in genes including enhancer of zeste 2 polycomb repressive complex 2 subunit (*EZH2*), lysine demethylase 6A (*KDM6A*), chromodomain helicase DNA binding protein 7 (*CHD7*), and zinc finger MYM-type containing 3 (*ZMYM3*) appear to disrupt chromatin marking of genes such as orthodenticle homeobox 2 (*OTX2*), *MYC* and *MYCN* in Group 3 and 4 tumors [31, 64, 128, 129].

Evaluation of genetic abnormalities and gene expression has revealed that Group 3 is most often associated with amplification and overexpression of *MYC* but not *MYCN* [19, 31]. While WNT tumors exhibit *MYC* amplifications, they also show amplifications in *MYCN* [26, 31]. Within the new Group 3 and 4 subtype classification system, Subtypes II and III have *MYC* amplification and are associated with the worst prognosis [35, 36]. While Subtype V consists of mostly Group 4 tumors, they exhibit amplification of either *MYC* or *MYCN* (Fig. 7).

Subtype I tumors are enriched for amplification of the *OTX2* oncogene and activation of growth factor independent 1 (*GFI1*) or *GFI1B* [35, 36]. Interestingly, it has recently been shown that OTX2 regulates MB stem-cell function in a subgroup-dependent manner [130, 131]. OTX2 plays an inhibitory role when overexpressed in SHH MB and is oncogenic in Group 3 and 4 MB. OTX2 promotes growth and self-renewal while inhibiting differentiation in vitro and increases tumor initiation from MB stem/progenitor cells in vivo [130, 131]. Further evaluation of the mechanisms regulated by OTX2 in Group 3 and 4 MB has provided a better understanding of the molecular signatures that contribute to pathogenesis of these highly aggressive subtypes. For example, Stromecki et al. [131] characterized the OTX2 regulatory network and identified novel relationships between OTX2 and genes associated with neuronal differentiation and axon guidance signaling in Group 3 stem/progenitor cells. This suggests that OTX2 actively represses differentiation while maintaining Group 3 cells in a primitive, stem/progenitor cell state. Additionally, Zagozewski

Subgroup		Group 3			
Subtype		I	II	III	IV
Demographics	Frequency (%)	4	13	9	10
	Most common age group (years old)	3-10	3-10	3-10	0-3 and 3-10
	Gender (% male : % female)	60% : 40%	77% : 23%	78% : 22%	68% : 32%
Clinical features	Histology	Classic > desmoplastic	LCA, classic	Classic > LCA	Classic
	Metastases	35	57	56	58
	Survival (at 5 years)	77	50	43	80
Molecular features	Driver events	- *GFI1* and *GFI1B* activation - *OTX2* amplification	- *MYC* amplification - *GFI1* and *GFI1B* activation - *KBTBD4*, *SMARCA4*, *CTDNEP1* or *KMT2D* mutation	- *MYC* amplification (less frequent)	None

Subgroup		Group 4			
		V	VI	VII	VIII
Demographics	Frequency (%)	8	9	22	25
	Most common age group (years old)	3-10	3-10	3-10	3-10 and 10-17
	Gender (% male : % female)	71% : 29%	67% : 33%	66% : 34%	75% : 25%
Clinical features	Histology	Classic	Classic	Classic	Classic
	Metastases	62	45	45	50
	Survival (at 5 years)	59	81	85	81
Molecular features	Driver events	- *MYC* or *MYCN* amplification	- *PRDM6* activation - *MYCN* amplification (less frequent)	- *KBTBD4* mutation	- *PRDM6* activation - *KDM6A*, *ZMYM3* or *KMT2C* mutation

Fig. 7 The Group 3 and Group 4 MB subgroups are currently divided into 8 different subtypes based on demographics, clinical and molecular features: Subtypes I, II, III, IV, V, VI, VII, and VIII. Adapted from Hovestadt et al. Nature Reviews Cancer, 2019

et al. recently identified another OTX2 regulatory network that controls the balance between the Group 3 MB stem cell state and differentiation [132]. They showed that OTX2 broadly restricts expression of transcription factors that are critical for neuronal differentiation, including members of the PAX gene family. They further identified mTORC1 signaling as a downstream effector of OTX2-PAX3, thus revealing a novel role for protein synthesis pathways in Group 3 MB tumor progression [132].

Group 3 and 4 tumors have a higher propensity to occur in males compared to females; however, the reason for this remains unclear. This may be partially explained by the three recurrently mutated genes, *ZMYM3*, *KDM6A*, and *DDX3X*, located on the X chromosome [64]. *ZMYM3* and *KDM6A* mutations are found almost exclusively in tumors from males [64, 133], while three out of four female MB patients carry a heterozygous mutation in *DDX3X* that escapes X inactivation

[64, 134]. In addition, 80% of all females with Group 4 tumors show a loss of the X chromosome within the tumor [19, 28].

Cell of Origin and Mouse Models of Group 3 and 4 MB

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 [135, 136]. Overexpression of MYC combined with *TP53* mutation resulted in highly aggressive tumors that histologically and molecularly resemble Group 3 MBs, albeit using different cells of origin. While Kawauchi et al. [135] overexpressed MYC in GNPCs, Pei et al. [136] 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 de-differentiated GNPC [135–137]. Indeed, studies by Hovestadt et al. [138] used scRNA-seq analysis to determine that Group 3 MB tumors predominantly consist of undifferentiated progenitor/stem-like cells. Similarly, Vladoiu et al. [139] also used single-cell transcriptomics to show that the different molecular subgroups of MB mirror the transcription programs from distinct, temporally restricted cerebellar lineage cell types. They determined that Group 3 MB tumors resemble Nestin+ stem cells [139].

It has been recently proposed that Group 4 MBs arise from progenitor cells of the upper rhombic lip [140]. Lin et al. [140] have shown that three master regulator transcription factors {LIM homeobox transcription factor 1 alpha (LMX1A), eomesodermin (EOMES), and LIM homeobox 2 (LHX2)} in Group 4 tumors exhibit overlapping spatiotemporal expression in deep cerebellar nuclei of the nuclear transitory zone. 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. Extending these findings, Hovestadt et al. [138] also analyzed Group 4 MB tumors using single-cell transcriptomes and determined that these tumors consist almost exclusively of more differentiated neuronal-like cells and resembled the unipolar brush cell (UBC) lineage, a glutamatergic neuronal cell population that arises from the upper rhombic lip. Similarly, Vladoiu et al. [139] also found that Group 4 MB are aligned with the UBC lineage.

While these cross-species comparisons in the mouse provided significant insight into the origins of Group 3 and Group 4 MBs, more recent and specific comparisons to the developing human cerebellum have revealed that these tumors predominantly arise from the rhombic lip subventricular zone (RL^{SVZ}), a region that is unique to the human cerebellum and not found in mice or macaques [141, 142]. These new and exciting findings suggest that humans are predisposed to the development of Group 3 and Group 4 MB and that there may be a window of opportunity to improve screening approaches for high-risk patients or to possibly start treatment at earlier stages of tumor development.

Treatment

Group 3 MBs are the most aggressive subgroup and exhibit frequent metastasis, making it incredibly difficult to treat these tumors. 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. *MYC* amplification provides a target for Group 3 MB treatment. Morfouace et al. [143] 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 [143].

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 subgroups. While there is currently no treatment targeting OTX2 specifically, studies have shown that the use of 9-cis-retinoic acid can reduce OTX2 expression and induces neuronal differentiation [144]. However, tumor cells quickly become resistance to retinoic acid treatment and different MB cell lines exhibit variable responses [145, 146]. 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 [147]. On magnetic resonance imaging (MRI), tumors are hypointense on T1 and hyper-intense on T2-weighted images and show marked contrast enhancement (Fig. 8). The number of patients with an atypical phenotype is low.

Perreault et al. [148] previously demonstrated that tumor location and enhancement patterns were correlated with specific MB subgroups 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 [149] or midline occurrence concomitant with dorsal brainstem infiltration [75]. The majority (54%) of SHH tumors were in the cerebellar hemispheres and this result was consistent with previously reported findings [149]. 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 [148]. 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 [148]. 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. However, there are additional layers of heterogeneity to consider, including the cancer stem cell hierarchy.

The cancer stem cell (CSC) model has evolved over the past two decades. Originally intended to explain the cellular and functional heterogeneity found between and within tumor subgroups, including MB, CSCs exhibit stem cell-like properties including self-renewal capacity and multi-lineage differentiation (Fig. 9) [150, 151]. These cells are operationally defined by their ability to regenerate an original tumor in xenograft serial transplantation assays. Thus, in theory, only the CSCs can create new tumors following long-term passage in immunodeficient animals. CSCs generate progenitor cells which are highly proliferative but have limited self-renewal capacity and are ultimately unable to maintain tumor growth

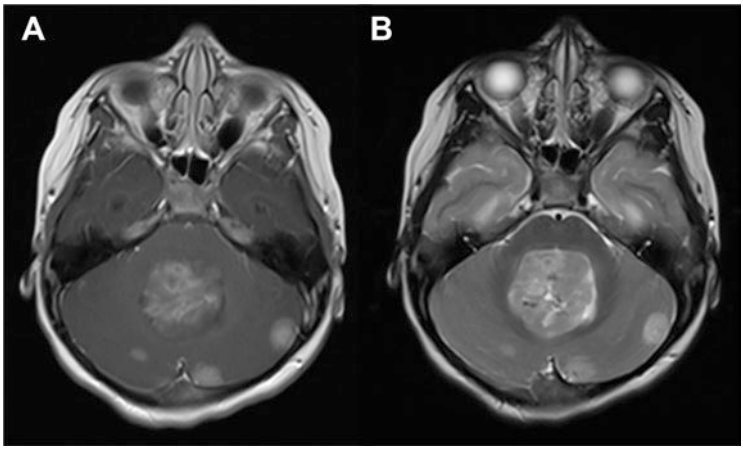

Fig. 8 5-year-old with multi-focal MB with a heterogeneous mass in the fourth ventricle and several other enhancing lesions in the posterior fossa. (**a**) T1 post-gadolinium image. (**b**) T2-weighted image

long-term. In addition to fueling tumor growth, CSCs have also been shown to drive therapy resistance through enhanced drug efflux and DNA repair [151, 152]. Despite the important clinical implications of CSCs, the model has generated substantial controversy over the years due to inconsistencies in CSC frequency, tumor-initiating capacity and proliferative potential as well as the methods and markers used to isolate putative CSC populations. While originally thought to be rare and quiescent, it has become increasingly evident that CSCs, and even progenitor cells, exhibit phenotypic plasticity in response to external stimuli from the microenvironment [151].

Singh et al. [153] were the first to demonstrate the presence of putative CSCs in MB. They further identified a cell surface marker CD133 (Prominin1), which selects for a highly self-renewing cell population in both MB and glioblastoma [153]. Subsequent studies provided further support for this model by demonstrating that CD133+ brain tumor cells from MB and glioblastoma patient samples were capable of initiating tumor growth when injected into NOD SCID mice. While CD133 is the most utilized BTPC marker, little is known about its biological function. These initial studies revolutionized the brain tumor CSC field. However, additional research determined that CD133 is not restricted to the CSC population as it was shown to be expressed in a variety of different cell types including normal stem cells and differentiated epithelial cells [154]. 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 [155, 156]. While Read et al. [155] demonstrated that Math1+/CD15+ neuronal progenitors are responsible for tumor propagation, Ward et al. [156] suggested that CD15 selects for a stem cell population rather than progenitor cells. More recent studies demonstrated that the stem cell marker Sox2 also plays a role in SHH MB tumor propagation [157, 158]. These authors showed that following treatment with chemotherapy and SHH pathway antagonists, the $Sox2^{+}$ cell population was enriched resulting in tumor growth and relapse [157]. 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 [155, 156], further work by Vanner et al. also showed that in order to reliably isolate the BTPC population, CD15 must be used in combination with Sox2. The potential clinical relevance of this SHH MB cell population is underscored by more recent findings demonstrating that specific subsets of stem-like cells within the Sox2+ cell compartment are resistant to treatment with the SHH-pathway antagonist vismodegib [159].

Recent studies have also identified a role for the low-affinity transmembrane neurotrophin receptor, CD271 (p75NTR), in regulating stem/progenitor cells in SHH MB [118, 119, 160]. Liang et al. have shown that CD271 expression is nearly exclusive to primary SHH MB patient tumors and functional characterization revealed a role for this cell surface marker in SHH MB tumor propagation and maintenance by modulating stem cell properties [118, 119]. Identification and functional validation of additional cell surface markers that select for MB stem cell populations in the different subgroups will undoubtedly provide further insight into the cellular complexity within these tumors. As recent studies have demonstrated that functional screening identifies more treatment options than MB sequencing

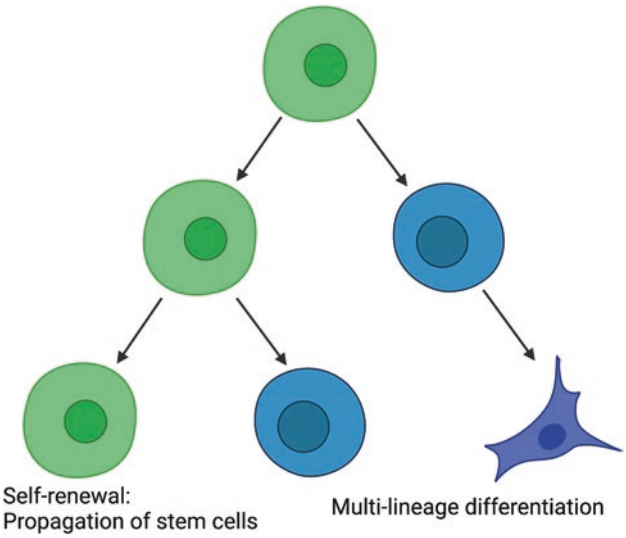

Fig. 9 Stem cells and cancer stem cells possess two characteristics, self-renewal and multi-lineage differentiation. Self-renewal is the ability to propagate oneself indefinitely and is a defining stem cell feature. Progenitor cells exhibit limited self-renewal capacity and ultimately differentiate. Self-renewal can occur as 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 but is 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. NB. Green denotes a stem cell, blue denotes further differentiated cells such as transit-amplifying progenitors

alone [161], this will require more extensive integration of bioinformatics data with functional assessment of relevant CSC signatures.

MB in the Molecular Era

MB currently represents one of the most extensively characterized cancers through large-scale bioinformatics analyses of patient cohorts. The extensive heterogeneity between and within the MB subgroups has led to the discovery of over a dozen molecular subtypes [33–35]. Consideration of these MB subtypes is becoming imperative to improve diagnosis and allow for selection of the most appropriate treatment regimens. For example, clinical trials have begun to implement molecular subgroup-informed strategies for treatment stratification [37]. Subgroup/subtype-specific therapies should be further explored by identifying and characterizing biomarkers that could ultimately lead to the development of novel diagnostic tools and targeted therapies. However, there are currently no molecular biomarkers to measure residual disease in children with CNS tumors, including MB. As a result, the

extent of tumor eradication cannot be assessed beyond the resolution of MRI. To address this challenge, multiple groups have recently explored the clinical utility of cerebrospinal fluid (CSF)-derived cell-free DNA (cfDNA) for monitoring and measuring residual disease in patients with MB [162, 163]. They found that CSF-derived cfDNA allows measurable residual disease detection and can predict treatment response in MB patients. These findings suggest that CSF-derived liquid biopsies should be incorporated into future trials.

Conclusions

MB research has significantly evolved in the past 10 years. Early gene expression array profiling studies led to the stratification of MB into 4 molecular subgroups. Further analysis of molecular features and clinical trends among these groups has resulted in additional substructure and classification of the MB subgroups into more than a dozen subtypes [33–35]. These studies, along with more recent work that resolved MB at a single cell level [76, 138, 139], have revealed the highly heterogeneous nature of MB that was once considered a single disease entity. From a clinical perspective, molecular subgrouping/subtyping has improved risk stratification and treatment options. However, despite concerted efforts to improve therapy, approximately 30–40% of patients still succumb to their disease while survivors are left with extensive cognitive and physical delays following surgery and treatment. The knowledge gleaned from over a decade of genomic, epigenomic, transcriptome, and even proteomic studies has paved the way for further functional studies that will fully characterize the mechanistic role of newly identified genes/pathways both in vitro and in vivo. This will ultimately lead to the development and implementation of innovative targeted therapies, including immune-molecular therapies via CAR T-cell-based approaches [164].

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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. Understanding the cerebellum's essential role in early neurodevelopment, and the association between cerebellar injury and long-term alteration in behavior is crucial. This chapter attempts 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

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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

Introduction

The cerebellum is traditionally considered the brain region involved in motor and non-motor activities [37, 101]. Given the major role of the cerebellum, in posture and movements, preliminary studies have shown that removal of this area can lead to impaired these activities [45]. These were in line with clinical reports that cerebellar degeneration may impair posture and speech, voluntary movement of extremities, and gait [50]. Several studies have been performed to understand the exact function of the cerebellum [51] and the importance of this area in controlling motor movements [104] and learning [54]. On the other hand, evidences have shown that extensive cerebellar connections to other areas of the brain (e.g., prefrontal and posterior parietal cortex) are associated with non-motor functions [21, 22]. Lately, imaging techniques have shown a link between cerebellar function and cognitive processes such as language [89], attention [2], and affective processes [43]. Therefore, it is believed that changes in the structure and function of the cerebellum can be attributed to several abnormalities in the emotional, cognitive, and social domains that are observed in patients with neurodevelopmental disorders such as autism spectrum disorders (ASD) and behavioral despair [73, 74, 95]. Consistent with the complex neurobiology of neurodevelopmental disorders and behavioral despair, the role of the cerebellum in non-motor functions should be well defined [8].

In this chapter, we provide a summary of the importance of the cerebellum in the pathophysiology of neurodevelopmental and behavioral disorders. Although the cerebellum has been shown to be involved in neurodevelopmental disorders, structural and functional differences in different regions of the cerebellum play an important role in attention-deficit hyperactivity disorder (ADHD), developmental dyslexia, and ASD. This suggests the hypothesis that the involvement of different cerebro-cerebellar circuits may lead to differences between the neurodevelopmental disorders [105]. In addition to these disorders, there are neurodevelopmental disorders such as developmental coordination disorder (DCD), which are often associated with the aforementioned neurodevelopmental disorders (e.g., ADHD and dyslexia) and hypothesize an association with cerebellar dysfunction [11, 130]. This information raises the question of how cerebellar dysfunction affects developmental processes and causes developmental disorders, and differences in the localization of cerebellar dysfunction may cause different disorders.

Cerebellum growth has been enormous during the first 24–40 weeks of pregnancy, resulting in approximately 5-fold volume and more than 30-fold in surface area [24, 121]. Cerebellar growth continues throughout the first postnatal year,

although neural differentiation and the growth of axonal inputs and outputs occur more slowly than in the prenatal stage [24, 121]. This process could interpret the fact that premature infants are at increased risk for cerebellar developmental disorder, hemorrhages, and future neurodevelopmental disabilities [24, 121]. 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 [98, 123]. The findings put the cerebellum at the center for neurological research into neurodevelopmental disorders, such as ASD [24]. Confidential evidence emphasized the obvious link between cerebral cortex injury in early life, which leads to an increased risk of affective and attention deficits, internalizing behavioral disorders, and withdrawal from social contact [77, 123]. Consistent with cerebellar tumor and/or resection of the tumor in children, an abnormal increase in the risk of cognitive and adaptive impairments [10], as well as the vermis injury, has been shown to be associated with long-term affective dysregulation [75]. It has also been shown that the vermis malformations are involved in higher rates of affective and behavioral disorders, including ASD [24, 111]. Congenital cerebellar malformations as well as types of early cerebellar lesions are directly related to ASD. To conclude from these findings, some scientists like Schmahmann et al. classified ASD as one of the psychiatric disorders associated with cerebellar damage or disease [97]. The studies have demonstrated that cerebellar injury in infancy is one of the main risk factors, which increases approximately 40-fold in developing ASD [77, 123]. Evaluation of various pathological conditions of the injured cerebellum has confirmed the association between injuries and ASD. For instance, tuber load in the cerebellum in children with tuberous sclerosis is considered a specific predictor of ASD [24, 124]. Cerebellar damage may cause some complications such as gaze aversion, stereotyped movements, linguistic impairments, as well as complete avoidance of physical contact, which eventually leads to ASD [93]. In line with the basic and experimental findings, the clinical evidence suggests that cerebellar injury at early stages through developmental diaschisis can affect the development of the cerebral cortical area to which the cerebellum projects [123]. 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.

Numerous studies in patients with ASD have reported abnormal changes in the size and shape of neurons in the deep cerebellar nuclei, as well as a decrease in the number of Purkinje cells (PC) [4, 60, 61, 85, 101]. Postmortem studies have confirmed experimental results showing a reduction in the gyrification, and size of granular and molecular layers of thee vermis, along with PC loss [8, 81, 110]. These findings may hypothesize that ASD is the source of prenatal defects, which persist in the early postnatal stage. Neuroimaging techniques, such as structural magnetic resonance imaging (MRI), provide conflicting information that shows vermal hypoplasia occurs in most people with ASD. The neuroimaging studies have also shown changes in the anatomical and functional connectivity of the cerebellum with other areas of the brain, including the thalamus and cerebral cortex [79, 101, 120].

In addition to the neuroimaging studies, pharmacological researches have shown that the cerebellar glutaminergic and GABAergic systems are targeted for dysfunction in ASD patients [12, 101].

Also, major psychiatric disorders, including major depressive and bipolar disorders, and schizophrenia, are presumed to have comprehensive changes in the GABAergic signaling system, such as altered cerebellar GABA receptor expression [101]. This could be associated with decreased FMRP expression and changes in FMRP–mGluR5 signaling and downstream targets, including RAC1, APP, STEP, and homer 1. On the other hand, GABA receptor expression is influenced by epigenetics or monoallelic expression. Thus GABAergic receptor agonism, modulation of mGluR5 activity, and inhibition of glutamate-induced excitotoxicity may be potential therapeutic strategies, along with drugs that affect monoamine systems, including dopaminergic or serotonergic pathways [39]. Indeed, the GABAergic system can be an important target for new drugs for psychiatric disorders [41].

Furthermore, papers on gene and protein expression analyses have demonstrated the downregulation of synaptophisin (SNAP-25, synaptosome-associated protein) and complexin, as well as the upregulation of semaphorin 3A, an axonal chemorepellant [35, 36, 82, 101]. Interestingly, dysregulation of activity and levels of D-amino acid oxidase (DAO), an enzyme that metabolizes D-serine, a co-agonist of NMDA (N-methyl-D-aspartate) receptor, were also observed [16]. Therefore, the available evidence seems to indicate disease-specific, including decreased vermis volume, and non-specific pathological factors, such as decreased PC count and pharmacological changes of the cerebellum in the neurodevelopmental disorders [101].

In addition to ASD, the cerebellum is involved in schizophrenia, demonstrating coordination and postural abnormalities, impaired eyeblink conditioning, and procedural learning deficits [63, 64, 103]. Neurological signs are thought to be related to structural changes in the cerebellum [8, 126]. Due to the extensive connections between the cerebellum and the forebrain regions, cognitive dysmetria and poor mental coordination have been suggested to be caused by cerebellar abnormalities in schizophrenic patients [6, 7].

Contribution of the Cerebellum in Neurodevelopment

There is growing evidence that emphasizes the role of the cerebellum in brain development. Studies in fetal, neonatal, and pediatric individuals support the hypothesis that the developing cerebellum is clearly involved in motor, cognitive, and sociobehavioral development, and exert the role associated with a regional functional topography of the cerebellum. Consistent with these data, investigational studies have indicated 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 brain development [106].

The developmental process of the cerebellum possesses a highly regulated pattern, which more rapidly grows during 20–40 weeks of gestation in comparison with other cerebral structures, demonstrating the importance of the critical period for cerebellar development [20, 78]. Cerebellar development in humans continues from the beginning of the first trimester to the end of the second postnatal year. However, prominent cerebellar development stage, including granule neuron progenitors (GNPs) proliferation, occurred in the last trimester [47]. Thus, cerebellar vulnerability and its developmental repercussions of injury can disrupt this highly orchestrated, programmed developmental process during a critical period. On the other hand, disruption of cerebellar growth significantly affects other areas of the brain, for example, the developing cerebral cortex [123]. The cerebellum makes up only 10% of the total volume of the brain, but makes up ~80% of the neurons in the brain [117, 122].

On the other hand, given the complexity of cerebellar development and the role of different genetic pathways in it (e.g., mutation of RELN gene induced cerebellar hypoplasia [52]), it is unlikely to impair its development. This is due to the rich interconnection of the cerebellum with different areas of the cerebral cortex that supports movement, cognition, and affective regulation [108]. In this regard, the cerebellum seems to play a modulatory role in cerebro-cerebellar circuits, and supports behavioral optimization, particularly in procedural learning and skill acquisition [106].

Subsequently, it is believed that early disruption of the cerebellum due to prenatal cerebellar developmental lesions (i.e., malformations), preterm delivery, 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, leading to long-term behavioral disorders [106].

Role of the Cerebellum in Adaptive Behaviors, Autism Spectrum, and Neuropsychiatric Disorders

It is clear that the removal of cerebellar tumor in children and cerebellar parenchymal injury in very preterm infants resulted in impairment of adaptive behaviors [10] and various types of affective disorders [75, 77]. For instance, affective dysregulation is associated with cerebellar dysfunction in children [75], while emotional lability is also observed following posterior fossa syndrome [90].

Regarding 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, 75]. Some reports have mentioned that most children with midline or vermal tumors are encountered to affect dysregulation [1]. These findings were supported by a study by Richter et al. [92] that both positive (e.g., reduced 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 attention to the critical role of the posterior vermis and its defects in neurodevelopmental disorders, including ADHD [57] and autism [9].

In addition, Schmahmann implied that more than half of the surviving preterm infants with cerebellar parenchymal tissue damage show psychiatric disorders [97], and functional limitations on socialization skills. Also, distinct socio-behavioral defects of attention, affective, internalizing, and pervasive sub-domains were reported in children with cerebellar injury [77].

Taken together, reports have shown that cerebellar injury and lesion at early life in preterm infants are associated with wide-ranging neurodevelopmental disorders [15]. Moreover, a reduction in the volume of the posterior vermis is thought to be consistent with neurodevelopmental-related behavioral dysregulation, including autism and ADHD. Psychiatric disorders have also been reported to be correlated with cerebellar injury during childhood [106].

Cerebellum Plays a Role in ASD

Evidence has proposed that dysfunction in specific areas of the cerebellum can result in neurodevelopmental disorders, including ASD, according to the cerebellum involvement in the developing brain. Scientists have demonstrated the significant role of cerebellar damage in the neuropsychiatric consequences in five main domains: (1) impairment of attention, and (2) emotion, (3) disruption of social skill, (4) psychosis, and (5) autism spectrum disorders [97]. In ASD, data strongly support the structural-functional abnormalities in the cerebellum in patients with autism. Although ASD is adjusted to result from cerebellar dysfunction, it is obvious that several brain regions 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 motor movements to optimize performance [55]. Also, it has been observed that activation patterns in the primary motor cortex are modulated by transcranial magnetic stimulation of the cerebellum [42]. This shows the cerebro-cerebellar relationship and verifies that alteration in cerebellar activity can affect different regions of the cerebral cortex, influence internal models of behavior, and optimize and predict future behavior [56]. Despite these effects, it does not mean that the cerebellar injury leads to complete loss of its function [95]. To this, a 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 motor movement but is associated with impairment of cognition and affect [56]. Moreover, there is region-specific motor dysfunction, as the posterior cerebellar injury demonstrates no severely impaired cognition and language, but it can lead to disrupted modulation and optimization of cognitive performance such as agrammatism or semantic fluency [95, 96]. 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 [30, 42]. A cerebellar role in learning and skill acquisition is compelling in neurodevelopment and neurodevelopmental disorders. Indeed, impairment of skill acquisition is more correlated to developmental disorders including ASD, dyslexia, and developmental coordination disorder [11, 115]. Several studies indicated that up to 40% of infants with cerebellar hemorrhages and lesions are diagnosed with ASD [117].

Another point to note is the sex difference in the prevalence of ASD. For every woman, three to four men are diagnosed. Due to the cerebellum's role in cognition and skill acquisition, it is a candidate to examine this sex difference. A study conducted by Smith et al. indicates a pattern of cortico-cerebellar hyperconnectivity in ASD females and a pattern of hypoconnectivity in ASD males [102].

A preprint study was conducted by Li et al. to establish a link between the clinical traits of ASD and the cerebellum. For this purpose, they performed amplitude of low-frequency fluctuations (ALFF) analysis. They found that the cerebellum but not other regions of the brain compared to normal controls showed significantly weaker average ALFF values [76].

These differences are assumed to be related to cerebro-cerebellar circuits [105]. Thus, behavioral defects resulting from neurodevelopmental disorders are linked to differences in the structure-function relationship of specific regions of the cerebellum [105]. 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 [109]. 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 the present findings, alteration in structure and function of the cerebellum is believed to the common phenomenon in ADHD [26, 32, 116], but the genetic and/or environment are thought to be predisposing risk factors of the neurodevelopmental disorder.

In children with ADHD the volume of the cerebellum, especially the gray matter in the left cerebellum is smaller compared to normal children ([65, 100]).

In a multicohort study, the role of cerebellar development in ADHD was investigated. The findings of this study showed that the growth pattern of cerebellum white matter in children with ADHD is slower during early childhood that was followed by faster growth in later childhood [100].

As well as the cerebellum's importance in ADHD pathogenesis, its correlation with other brain regions maybe have a prominent role. Ding and Pang indicated

strong functional connections between the cerebellum and the left, right middle frontal gyrus and the left parahippocampal gyrus, in comparison to the control group [29].

One of the drugs used in the treatment of ADHD is methylphenidate, which is able to improve T2 relaxation time in the cerebellar vermis in ADHD children [5]. Also, a systematic review study demonstrated that the cerebellum besides the middle and inferior frontal gyri and basal ganglia were most often affected by methylphenidate administration in ADHD patients [23].

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 the incidence of ADHD [68, 84]. 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 [32, 70, 71]. XK-protein is observed to be widely overexpressed in the brain compared to Kell-protein in the Purkinje cells of the cerebellum. 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 [25] 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 [114], while an SNP in the XKR4-gene has contributed to responsiveness to antipsychotic therapy [40, 69].

Recently, it has been shown that there is a significant relationship between MANBA gene (encoding for β-mannosidase) expression in the cerebellum and ADHD risk. As a result of rs1054037(C > T) mutation, and elimination of the binding site for hsa-miR-5591-3P, MANBA gene expression was upregulated [18].

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 [33]. Moreover, in contrast to some reports suggesting that the cerebellum's heritability may be enhanced into adolescence and adulthood [88, 118], the cerebellum is considered as the least heritable brain structure at birth [44] and in childhood [87]. Prenatal adversity may influence cerebellar development, which begins in early intrauterine life [78, 112, 113]. These show the importance of prenatal and early postnatal periods in the development of the cerebellum to reach a normal structure and function. Unless, negative effects on the cerebellum in patients with ADHD have been demonstrated to be relevant to impairment of the cognitive phenotypes, such as temporal processing [34]. However, the role of environmental effects on cerebellar development and its contribution to the symptoms of neurodevelopmental disorders remained to be obviously understood.

Cerebellum Plays a Role in Behavioral Despair and Neuropsychiatric Illnesses

Body of evidence 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 the dorsolateral prefrontal cortex (DLPFC), anterior cingulate cortex (ACC), and hippocampus was observed in patients with MDD [14, 28, 31, 66, 129]. The reports have suggested two disorders as the pathophysiological factors of MDD, as below:

- Impairment of structure and function within cortico-limbic circuitry [58].
- Alterations in the functional organization of multiple brain networks are implicated in attention regulation, emotional processes, and cognitive control [58].

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 the linkage of the cerebellum with cerebral cortices and paralimbic regions. Indeed, corticocerebellar circuits are the key point to clarify the role of the cerebellum in MDD [97, 107]. Limited data on the involvement of the cerebellum in MDD may be related to the few studies of cerebellar structure in MDD.

Cerebro-cerebellar circuits have been affected in MDD. He et al. evaluated this circuit in MDD and BD (bipolar disorder); their results demonstrated weaker negative and positive functional connectivity in the cerebro-cerebellar affective and cerebro-cerebellar default mode networks, respectively. However, connectivity within the cerebro-cerebellar default mode network in MDD patients was weaker compared to the BD patients [49].

However, the analytical studies were focused on the vermian volume and lack of gray/white matter parcellation [127]. Moreover, clinical evidence reported an abnormal structure of the cerebellum in depressed patients using whole-brain investigations of altered GMV in depression [28, 67, 86, 119, 127].

In addition, in remitted MDD (rMDD) patients with cognitive deficits, GMV significantly lowered in left area VIIA, crus II, and in vermal area VIIB in comparison to healthy controls. Depping et al. based on their findings suggest that the VII area in the cerebellum can be targeted to treat cognitive deficits related to MDD by non-invasive brain stimulation [27].

In another study, Bogoian et al. looked at the link between depressive symptoms dimension and the cerebellum in late life. There is a positive relationship between depressive symptom profiles severity and cerebellum especially vermis volume (VI and VIII) [13].

In a study, self-perception of negative mood was evaluated in patients with cerebellar damage mostly posterior vermis, that allows conscious emotional processing. Their results showed that this damage might slow the data integration for mood state awareness. Authors believed it is one of the reasons for the underdiagnosis of depression in cerebellar patients [19].

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 [91]. 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 [53].

There are various reports that confirm the alteration of cerebellar-default-mode network (DMN) connectivity in OCD [80, 83].

Several studies sought to establish an association between the cerebellum and functional networks in the pathogenesis of OCD. Zhang et al. highlight functional connectivity between the cerebellum and the cortico-striato-thalamo-cortical (CSTC) circuit in OCD patients. The mean amplitude of low-frequency fluctuation (mfALFF) values significantly increased in the cerebellum of OCD patients compared to healthy controls. Their hypothesized, weak functional connection between the cerebellum and CSTC may be involved in the pathogenesis of the OCD [128].

In another study, Sha et al. demonstrated that the cerebello-thalamo-cortical network is functionally disrupted in OCD patients. Their results exhibited lower connectivity within the somatomotor network (SMN) and greater SMN-subcortical network and SMN-cerebellar connectivity in OCD [99].

Although we have limited data on the role of the cerebellum in the pathogenesis of anxiety disorders, the 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].

In a study, Sakakibara et al. sought to evaluate the resting-state activity of the cerebellum and its correlation with trait anxiety and parenting stress. It was found in mothers with less adaptive sensory processing, resting-state network activities significantly increased in the left lobule VI of the cerebellum [94].

Anxiety is one of the most frequent psychiatric illnesses in adolescents. Lee et al. evaluate functional connectivity alteration in the cerebellum and its relation with anxiety in Adolescents. Dentate nuclei communicate the cerebellum with cortical regions. They highlighted alterations in this area during anxiety in patients and found significant hyperconnectivity between salience-motor Dentate nuclei functional territories and cerebral cortical salience-motor regions compared to controls [72].

Schizophrenia, as known as a 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 impaired cognition, termed “cognitive dysmetria” that involves the thought-form. The literature proposed that this condition may be relevant to the pathological status of the cerebellum [125]. 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, 59].

The functional connectivity between the cerebellum and cortical/subcortical network is disturbed in schizophrenia. Reduction in the gray matter of the cerebellum during schizophrenia increased static and decreased dynamic functional connectivity between the cerebellum and cortical/subcortical networks, respectively [48]. In another study on schizophrenia patients, significant hypoconnectivity was shown between the cerebellum and cortical resting-state network. They suggest impaired resting-state functional connectivity in specific lobules of the cerebellum, could be a biomarker for schizophrenia [62].

Cai et al. in their study evaluated the association between cerebellar-cerebral resting-state (rsFC) functional connectivity and neurological soft signs. Results of this study demonstrate that in schizophrenia patients, uncoupling of rsFC between the cerebellum and the cerebral cortex may induce the expression of neurological soft signs. They concluded that in these patients, cerebellar-prefrontal rsFC has a positive correlation with both motor coordination deficits and negative symptoms [17].

The results showed a decrease in the volume of the cerebellum and cerebrum in adolescent patients with schizophrenia. Moreover, Greenstein et al. [46] explored abnormal different trajectories of cerebellar development in patients with childhood-onset schizophrenia.

Conclusion

The body of evidence has shown a critical role of the cerebellum in the development of motor and non-motor (e.g., cognition and behavior) conditions, that were disrupted by cerebellar injury in preterm infants, developmental cerebellar lesions in infants, cerebellar tumor in pediatric patients, and neurodevelopmental defects. As developmental differences have occurred in cerebellar malformations and neurodevelopmental disorders, it is thought to be associated with motor, cognitive, and behavioral dysfunction. Cerebellar injury in preterm infants can increase the rate of cognitive and socio-behavioral dysfunction. Consistent with preterm newborns, cerebellar tumors 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 neurodevelopmental and behavioral disorders. Cerebellar dysfunction in early life can cause distinct, long-term effects on the brain distal areas which are projected by the cerebellum. Developmental diaschisis can affect the structure-function of the areas of the cerebral cortex that may be optimized by the cerebellar input. In summary, increasing clinical and neuroimaging evidence in newborns with acquired and developmental cerebellar lesions, along

with older children with cerebellar damage, provided a new approach to the role of the cerebellar lesions in early life on cerebral development. On the other hand, determining the age of cerebellar injury to a developing brain may help us predict 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 way to explore a new treatment for cerebella injury-induced neurodevelopmental and behavioral disorders caused by cerebellar neuromodulation. It is also possible that therapeutic interventions, such as cerebellar neuromodulation, may offer alternative treatment options in these populations. Growing our knowledge of the association between cerebellar circuits and specific behaviors can

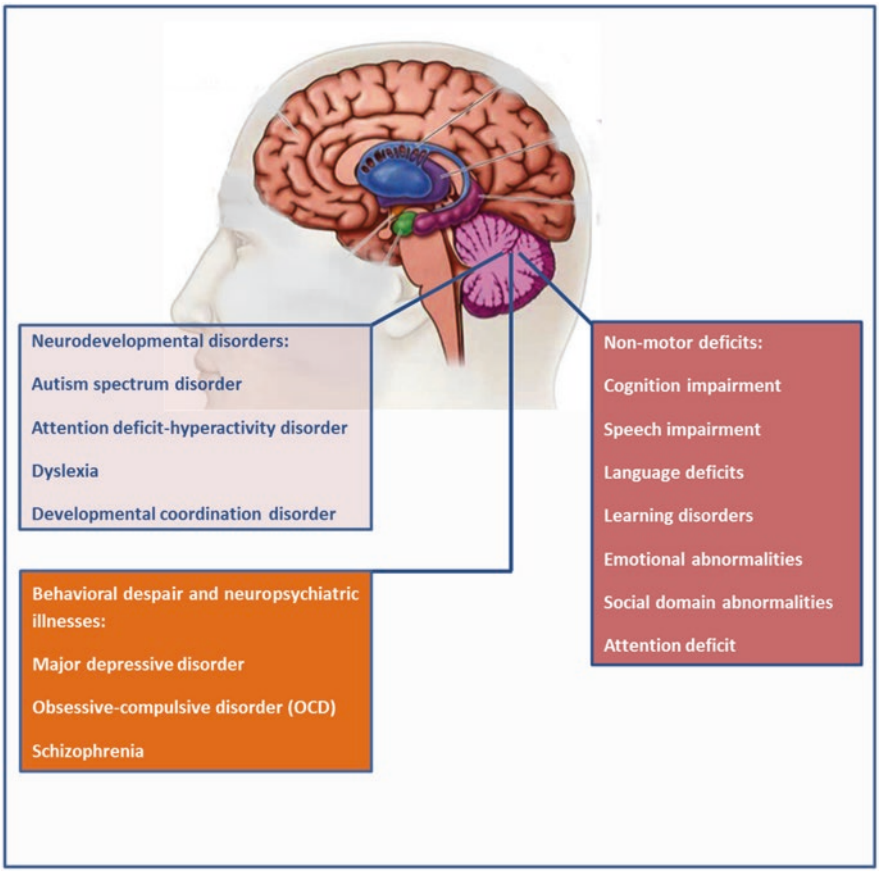

Fig. 1 Schematic of the cerebellum and its associated non-motor, neurobehavioral, and behavioral disorders. The cerebellar damages and dysfunction may lead to a variety of non-motor deficits and behavioral outcomes in patients with neurodevelopmental disorders

facilitate reaching to point of optimization of timing and localization of the therapeutic strategies. These essential findings will guide us to improve the lives of millions of children affected by cerebellar injury and subsequent developmental 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 crucially 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 contribute to cerebellar deficits in ASD patients. Structural and functional cerebellar abnormali-

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ties 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

Introduction

Autism is a complex neurodevelopmental disorder that was described as "early infantile autism" for the first time by Leo Kanner, a child psychiatrist (1943). He used this term for patients with "a powerful desire for aloneness" and "an obsessive insistence on persistent sameness" [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, Fifth Edition* (DSM-5) collectively designated all autism-like disorders as "autism spectrum disorder" (ASD) [3].

Despite the fact that the symptoms of ASD are extremely different, it 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]. Additional conditions that can be associated with ASD are psychiatric disorders such as attention deficit hyperactivity disorder (ADHD) and genetic defects [5]. Current diagnostic methods can detect autism in children as young as 2 years old [11]. It is estimated that 1 in 68 children in the United States has ASD [12], and 1–2% has the disorder in Asia, Europe, and North America (see chapter "Epidemiology of Cerebellar Disorders"), as well as male children are four times more likely to be diagnosed with ASD than female children [11]. Part of the reason for this ratio in the diagnosis of autism is further complicated by co-occurring conditions such as depression, sensory problems, and seizures in women that lead to misdiagnosis of ASD at a young age [13].

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, 14].

A subset of ASD patients, about 1 in 5, displays increased head circumference and brain volume in early childhood, typically until 5–6 years of age [15, 16]. These patients have a greater cerebral white matter, a thicker corpus callosum, and a higher volume of cerebrospinal fluid (CSF) in the subarachnoid space from 6 to 9 months of age [17–19]. The frontal cortex has been reported to be larger, probably due to increased neuronal density in the prefrontal cortex [20]. Other brain regions that are prominently implicated in ASD include the cerebellum, brainstem, and limbic

system, including the hippocampus and basal ganglia [21]. 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 [22]. Additionally, enlargement of the amygdala and caudate nucleus may cause anxiety and repetitive behavior [23].

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, 13, 24, 25]. The cerebellar neurodevelopmental deficits in ASD include increased size of the cerebellum (increased cortex in lobule V); reduced cerebellar cortex, especially lobule VIII, right Crus I, and midline IX; decreased size in vermal lobules VI and VII; and abnormal cerebellar circuits with rostral part [13]. Neuronal malmigration may cause cortical distortion and the presence of ectopic neurons in the white matter [26], neurodegeneration, and impaired cerebellar circuits. Together, these deficits affect motor, sensory, language, and cognitive functions [27–30].

Autism Spectrum Disorder Pathogenesis

Emerging evidence from genetic association studies and post-mortem 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. Autism affects a large number of biological processes, brain networks, and behaviors. As a result, it has been difficult to uncover the neurobiological underpinnings of ASD [9, 31] and in what ways 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 cerebellum susceptible to many risk factors [32–36]. 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 "Epigenetic Control and Cerebellar Neurodevelopmental Disorders".

Genetic Factors

Several lines of evidence have revealed that ASD is a neurodevelopmental disorder determined largely by genetic factors [37]. 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% [38]. Recently, various genes have been discovered as risk factors for ASD in genome-wide association studies. These genes, which span several chromosomal loci, are highly expressed and involved in the development of the cerebellum [39, 40]. Sadakata et al. [40] categorized these genes based on their role in the 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, and leucine-rich repeat transmembrane neuronal proteins (LRRTMs), Shanks and SynGAP [41–44]. Another category of ASD risk genes, such as EN2, TSC1, FMR1, and MECP2, encode for proteins necessary for transcription and translation [40, 45].

Chromodomain-helicase-DNA binding protein 8 (CHD8), previously called Duplin, is one of the genes most strongly associated with ASD [46, 47]. CHD8 is a chromatin-remodeling factor that is contributed to a variety of biological processes such as cell cycle, cell adhesion, development of neurons, myelination, and synaptogenesis [48]. CHD8 is an ATP-dependent chromatin-remodeling factor [49] and may serve as a "master regulator" for other ASD risk genes during fetal development [46, 50]. Knockdown of CHD8 in human neural stem cells affects the expression of several ASD risk genes [46], and human patients with mutations in CHD8 display ASD symptoms and have macrocephaly and gastrointestinal difficulties [39]. Furthermore, in mice, cerebellar granule neuron progenitor (GNP)-specific deletion of CHD8 affects cell proliferation and differentiation, as well as causing cerebellar hypoplasia and a motor coordination deficit [51]. These findings suggested that CHD8 regulates other ASD risk genes and targets a collection of genes throughout brain development [46]. Some of the ASD risk genes regulate developmental processes in the cerebellum [14]. These include genes encoding for Reelin, RORα, EN2, BDNF, neuroligins, and neurexins [8, 52].

RELN dysregulation has been observed in a subset of autistic individuals (reviewed in [38, 52]). Reelin, encoded by the RELN gene (located in 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, 38]. 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, 52]. Persico et al. first reported that the polymorphic GGC repeats located in the 50 untranslated region (50 UTR) of the RELN are associated with ASD disorder [53]. The finding was subsequently replicated in three studies: Zhang et al. [54], Skaar et al. [55], and Dutta et al. [56], 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 causes ASD, particularly in patients with speech difficulties [57]. Several RELN genetic variants including triple replication of 5′UTR CGG and polymorphisms rs736707, rs362691, and rs2229864 were examined. No significant associations were observed between allele frequency or genotype of the studied polymorphisms and triple replication of 5′UTR with ASD [58].

Reelin mutations in mouse models lead to irregular cortex formation and abnormal layering, which may responsible for behavioral and neurological disorders [59]. Adulthood changes in Reelin protein level caused cognitive impairment and reduced synaptic plasticity [59–62]. 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 [63]. A study by Cuchillo-Ibáñez comparing plasma Reelin levels in children with autism and healthy children of similar ages, in both sexes, showed that Reelin expression was 30 times higher in half of children with autism than in non-autistic individuals. This protein was shown to be significantly higher in boys with autism than in girls [64].

Several lines of evidence indicate that genes encoding retinoic acid receptor-related orphan receptors (RORs) are also associated with ASD. The RORα, β, and γ are nuclear receptors regulate a range of physiological processes during brain development [65–67]. RORα and RORγ are broadly expressed in the body, whereas RORβ expression is more restricted to the central nervous system [67, 68]. RORα protein expression significantly decreases in the brains of ASD patients probably through epigenetic alterations [69]. Devanna and Vernes demonstrated that miR-137, a microRNA implicated in neuropsychiatric disorders, targets a number of genes associated with ASD including RORα [70]. RORα is a transcription factor that is critically important for development of the cerebellum [65, 66, 71]. The role of the RORα in neural development has been demonstrated in mouse strain *staggerer*, which harbors a spontaneous deletion within RORα [72]. 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 [72]. 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 [66, 67].

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 [73, 74], which are both associated with susceptibility to ASD [75].

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 [76]. Although there is no consistency in the association between BDNF levels in blood and ASD, a recent review by Qin et al. using meta-analysis indicated that there are increased peripheral blood levels of BDNF in ASD patients [77]. Increased platelet counts in autistic children could provide a clue as to why BDNF levels are increased, since these are the most important peripheral reservoirs for BDNF [78]. Furthermore, Ca_2^+-dependent activator protein for secretion 2 (CADPS2) contributes to normal cerebellar development by enhancing release of BDNF and neurotrophin-3 (NT-3) [79, 80]. 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 [40, 81].

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 [82], which is under the umbrella of ASD. Patients with Rett syndrome frequently have cerebellar atrophy that increases with age [14] (see chapter "Epigenetic Control and Cerebellar Neurodevelopmental Disorders").

Tuberous sclerosis complex (TSC) is a genetic disease that causes benign tumors in the body, including brain [83]. Mutations in the TSC1 and TSC2 genes cause TSC with a neurodevelopmental disorder that involves higher rates of ASD [83, 84]. TSC produces a protein that negatively regulates the target of the rapamycin (mTOR) signaling pathway to control molecular and cellular process. Tsai et al. designed a mutant mouse model in which the gene for Tsc1 is not expressed in Purkinje cells [84]. These mutant mice displayed ASD-like behaviors such as abnormal social interaction, ultrasonic vocalization, and inflexibility. In addition, recent discovery have shown that the granule cells/Purkinje cells are important for cognitive processing in the cerebellum [85]. These studies are significant because they demonstrated a clear involvement of the cerebellum in nonmotor functions as well [84].

The Role of Glia

There is growing evidence that glia cells have been implicated in pathophysiology of ASD. Glia cells play a key role in developing synapse, myelination, neurogenesis, and inflammation within the brain. Autism-related neurogenesis and synaptogenesis deficiencies suggest that glia cell dysfunction may contribute to the development of autism or can play a dual role in improving or worsening ASD symptoms (reviewed in [86]).

Molecular studies on autism-related genes indicated a link between ASDs and genes involved in glial cell activation. Increased number of glia cells and activation of microglia in different areas of the brain including the cerebellum, as well as increased expression of proinflammatory factors such as cytokines, were observed in brain samples of people with ASD using PET scan and post-mortem brain samples [87–89].

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, Bisphenol A [90], gestational diabetes [91], infection with viruses (e.g., influenza, rubella, and cytomegalovirus)) during pregnancy, and advanced age of parents (for reviews, see Refs. [40, 92, 93]). Additional factors such as zinc

deficiency, abnormal melatonin synthesis, and prenatal stress may also contribute to autism [93].

Some environmental risk factors, such as prenatal valproic acid exposure, have been linked to aberrant cerebellar development and ASD [94]. In rat, valproic acid exposure reduces the number of Purkinje cells in the cerebellum accompanied by increases in the number of apoptotic cells [95]. Cole et al. have shown changes in cerebellar gene expression in mice treated with chlorpyrifos [96]. Dermal exposure of young adult mice to chlorpyrifos causes increased glial fibrillary acidic protein expression of the cerebellum [97]. Furthermore, Purkinje cell numbers are reduced in rats prenatally exposed to chlorpyrifos [98]. Other factors such as organophosphate pesticides and antiepileptic drugs have been shown to affect cerebellar development and potentially cause ASD [99]. 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 [100–102]. The risk of diseases such as preeclampsia, fetal macrosomia, perinatal mortality, caesarean delivery, and preterm childbirth is higher in women with gestational diabetes, which can increase the risk of developmental neurological disorders and ASD [103].

Viral infections can affect cerebellar and neocortical development during pre- and neonatal and cause neuropathy in ASD [104, 105]. 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 [106–109] (see chapter "Infections of the Cerebellum").

Covid-19 and ASD

A group of evidence indicated that deficiency of insulin-like growth factor-1 (IGF-1) seen in newborns of women suffering from Covid-19 may plays a vital role in the etiology of ASD [110–112]. In a Covid-19 condition, maternal immunologic activation elevates interleukin (IL-6), which lowers growth hormone and IGF-1 synthesis in the placental environment. It is suggested that IL-6 causes decreased Covid-19 infection resistance due to suppressed IGF-1, which is typical in older people. The ability of the developing nervous system of the fetus to myelinate would be damaged and leading to brain connectivity impairment [110, 112]. This could increase autism in the newborns of pregnant women who are currently suffering from Covid-19 [110].

Functional Gastrointestinal Disorders and ASD

Functional gastrointestinal disorders (FGIDs) are disorders independent of organic or physiological conditions that are the most common causes of gastrointestinal disorders in children with ASD. FGID symptoms include abdominal pain,

constipation, irritable bowel syndrome, and functional dyspepsia [113]. The FGIDs are associated with impaired behaviors and sensory responses and changes in sleep patterns [114]. Because many autistic children have co-occurring gastrointestinal disorders, new research suggests a probable relationship between ASD and the gut microbiome (reviewed in [115]). It is suggested that inadequate brain–gut interactions may be responsible for these symptoms in ASD patients [113]. 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 [116, 117].

Air Pollution and ASD

Exposure to air pollution, which may cause immune response, is another likely environmental risk factor for ASD [118]. A maternal illness with a fever during the second trimester of pregnancy increases the infant's risk of developing autism after birth [119]. The immune response results in the 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 impairs cerebellar morphology and various motor and nonmotor behaviors [120]. The abnormal level of immunological markers in the blood of ASD patients is shown to be evidence of interaction between genetic/environmental factors and the immune system in these patients [121, 122] (see chapter "Interrelation Between the Immune and the Nervous Systems in the Context of Cerebellar Development and Developmental Disorders").

Diagnosis of ASD

Studies on patients with ASD using advanced 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 the current clinical diagnosis of these patients is based on behavioral observations combined with patient history [23, 123]. 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 [123]. Murphy et al. highlighted three key issues regarding physical health that may be important in the diagnosis of ASD patients: sleep, gastrointestinal problems, and epilepsy [123]. The mental health issues are present in adults and children with ASD, including 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 [123–125].

The Cerebellum and ASD

Cerebellar abnormalities have been linked to a variety of developmental disabilities and behavioral disorders, including ASD (see review by Bolduc and Limperopoulos [33]). Vakorin and colleagues employed resting-state magnetoencephalography to calculate resting spectral power and interregional synchronization in early and late childhood of people with and without ASD. They found that the frontal cortex and cerebellum play a prominent role in ASD [126]. Motor impairment and clumsiness have been noted as essential features of ASD [127]. It is shown that about 80% of children with ASD have motor coordination deficits, which positively correlate with the severity of the ASD and intellectual disabilities [13, 128]. 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 [13, 129]. Motor impairments are among the earliest signs of an autistic phenotype [130]. 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, as well as language development delays [13, 131].

Similarly, difficulties in oral and manual motor skills in infancy can label individuals as ASD patients, and late speech fluency is predictable [132]. In addition, early motor delays are more common in infants at risk for ASD and are related to later communication delays [133]. 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 motor task performance in ASD patients [134]. The lack of gesture and imitation in ASD patients has been linked to motor dysfunction, suggesting a method through which cerebellar impairment could affect the core social communication symptoms in ASD patients [13, 135].

ASD and Cerebellar Structure Abnormalities

Cerebellar abnormalities such as reduced white matter integrity [136], abnormal dentato-cerebral functional connectivity [137], abnormal gray matter volume in the cerebellar cortex [138], and decreased cerebellar cortex (which is a key landmark for diagnosis in ASD brains) are the most consistently reported brain structural changes in ASD [13, 139]. Cerebellar enlargement has been reported in ASD young children compared to the total brain volume and may be associated with the

cerebellar white matter [28, 140, 141]. However, the growth rate declines later during development and eventually results in a smaller cerebellar volume by adulthood in ASD patients [141, 142].

MRI reveals hyperplasia and hypoplasia (with hypoplasia being more common) in the cerebellar vermal lobules VI and VII of patients with ASD, with possible origins from environmental trauma or genetic factors [143, 144]. It is suggested that these alterations may be responsible for increased stereotypes and repetitive movements [145]. 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 [146, 147]. A voxel-based morphometry study 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 [13, 148].

Neurohistological studies show changes in the anatomy of the cerebellum in patients with ASD, including a decrease in the number of Purkinje cells [149, 150], immature cerebellar development [151–153], morphological changes in the size of the cerebellar nuclei which are small and abnormal, and an increase in the number of Bergmann glial cells [154, 155]. 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 [156]. Small-sized Purkinje cells may indicate an atrophic process [157]. Purkinje cells have a high metabolic demand because of their large size and numerous synapses with parallel and climbing fibers. Therefore, they have extensive amounts of calcium storage that may cause increases in intracellular calcium, which elevates the risk of excitotoxicity and cell death [158].

It has been reported that the cortico-ponto-cerebello-thalamo-cortical 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 related to the neocortical areas are unusual in ASD patients [159, 160]. The cortico-ponto-cerebellar pathway carries inputs to the cerebellum from the primary sensory and motor cortex, posterior parietal, prefrontal, orbitofrontal, cingulate, temporal, and basal nuclei [161, 162]. Outputs originate from the cerebellar nuclei and project to the neocortex through the thalamus [163–165]. These circuits are specialized for cognitive and behavioral functions such as executive functions, language, and emotions. Thus, the cerebellum may be responsible for cognitive impairment, sensorimotor behavior, and social disconnection in ASD [14].

Eye-gaze abnormalities during social interaction are early diagnostic indicators 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 [166]. The pontine nuclei stimulate Purkinje cells in lobules VI–VII vermis cerebellum, and inhibitory outputs from the oculomotor vermis stop undesired eye movements and keep an image on the fovea [167], which could potentially be used as an early marker of ASD patients.

Control of upper limb movement is related to the frontoparietal cortex and the cerebellar cortex, as well as its output nuclei [168]. In patients with upper limb ataxia, atrophy of the intermediate and lateral cerebellum involving lobules I–V and more lateral portions of lobules V–VI extending into Crus I–II is linked to upper limb and manual motor impairments [169]. These loops regulate the amplitude, duration, and timing of movements [170, 171]. Patients with ASD have difficulties coordinating grasping and reaching activities [172]. Central defects may cause these difficulties in integrating sensory feedback information, motor output, and 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 and 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 [173]. The motor deficits start from infancy and extend to adolescence and adulthood [127, 174–176].

Cognitive function deficits such as attention and memory impairment, executive function, and cognitive flexibility deficits are common features in ASD [177]. 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 [178–180]. The cerebellum to prefrontal cortex pathway could directly or indirectly affect cognitive functions through the ventral tegmental area, which contains dopaminergic neurons that project and terminate in the prefrontal cortex [181]. Notably, the function of the prefrontal cortex dopaminergic pathway is associated with attention selection, cognitive flexibility, and memory [180]. A maldevelopment and atypical connectivity of the cerebellum to this higher order circuit may explain the cognitive involvement of the cerebellum in patients with ASD.

The brain connectome reveals the structure and configurations of the brain in terms of its spatial and temporal alternation. The brain connectome can change at any moment during life due to neurodevelopmental diseases such as ADHD, ASD, or other neurodegenerative disorders in the early stages of development [182]. Although no functional brain connectome map for ASD exists now, there is an agreement based on biological characteristics that the ASD connectome reveals ectopic and immature connections [183], which could be the outcome of abnormal brain development. Moreover, these aberrant functional connectivity patterns were found to be substantially linked to the severity of ASD symptoms [184, 185]. In ASD, there have been reports of both hyperconnectivity and hypoconnectivity patterns, which could result from hormonal changes in developmental growth during puberty that alter neural connections and function [186].

Assessment and Treatment

There is minimal accurate and practical information to assess, diagnose, and manage ASD conditions. Therefore, because the number of ASD patients has rapidly increased during the past decade, there is an urgent need to improve knowledge, develop assessment tools, and treat ASD patients [123].

ASD diagnosis can be difficult because of heterogeneity, varying presentation, and variability in symptoms [187]. There are no biomarkers to diagnose ASD. Therefore, the behavioral presentation of the patient is used for diagnosis [188]. The gold standard for clinical diagnosis in these patients is based on current diagnostic classification systems and 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 entire developmental history [188]. Currently, the best practice to diagnose ASD patients is the step-by-step strategy recommended by the American Psychological Association [189]. This diagnostic strategy starts with the child's parent/caregiver concern and is 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 and analysis/assessment of behaviors based on the developmental aspect [190]. However, because of differences in cognitive function, age, language level, and the source of information, diagnosis of ASD is very difficult [187].

Children diagnosed with ASD need to be reevaluated continuously during preschool years to identify their weaknesses, inabilities, and difficulties [187]. There are also some diagnostic instruments for ASD, such as the Autism Diagnostic Observational Schedule – Generic (ADOS-G) [191], 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) [192] 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) [193]. Additionally, there is a clinical diagnostic instrument named the Autism Diagnostic Interview – Revised (ADI-R) for the parent interview that addresses early development, communication/language, social interactions/interests, and restricted and repetitive behaviors [194]. The Social Communication Questionnaire (SCQ) is an appropriate method to get information from parents [195].

Usually, an assessment starts with a medical evaluation conducted by physicians. If the ASD is suspected, the patient is referred for diagnostic assessment by the pediatrician. When the diagnosis is confirmed, treatment planning should involve the professional health team [187].

Summary

Many genetic and environmental factors may cause ASD. The mechanisms are unknown, but presumably, genetic and environmental factors affect normal brain development and lead to functional disorders in patients with ASD.

There is mounting evidence that developmental abnormalities in the cerebellum may underlie the pathogenetic mechanisms associated with the ASD phenotype. Cerebellar developmental disorders associated with ASD pathogenesis show deficits in motor coordination, balance, motor memory, and higher order dysfunctions, including speech and attention regulation.

The primary goal of management in ASD patients is an 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 gastrointestinal 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 health-care strategies for 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 technologies such as magnetic resonance imaging (MRI), which has revealed many developmental disorders of the cerebellum. These malformations cause impairments that affect motor and nonmotor functions. Cerebellar hypoplasia (CH), cerebellar dysplasia (CD), Dandy–Walker malformation (DWM), Joubert syndrome and related disorders (JSRDs), pontocerebellar hypoplasia (PCH), rhombencephalosynapsis (RES), lissencephaly with cerebellar hypoplasia (LCH), and Lhermitte–Duclos disease (LDD) are examples of cerebellar malformations which this chapter will focus on using characteristic symptoms and signs. The current approaches for evaluation of the affected patients, differential diagnosis, and management of the malformations will be discussed.

Keywords Cerebellar hypoplasia · Cerebellar dysplasia · Dandy–Walker malformation · Joubert syndrome · Pontocerebellar hypoplasia · Rhombencephalosynapsis · Lhermitte–Duclos disease

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Introduction

The cerebellar development and structure have been discussed in chapter "The Embryology and Anatomy of the Cerebellum". The cerebellar development begins during an early embryonic stage with a complicated developmental process that continues well into the first year after birth in human. Recent advances in neonatal intensive care and breakthroughs in neuroimaging techniques such as positron emission tomography (PET), structural MRI (sMRI), and functional MRI (fMRI) have improved our ability to understand the structural and functional anomalies that implicate cerebellar involvement in numerous motor and nonmotor functions, ranging from motor/sensory integration and working memory to various higher order cognitive processes [1–4]. Despite the advanced technologies, understanding cerebellar malformations in children requires additional research regarding their prognosis as well as their lifelong consequences. Because of a lack of an 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 and environmental factors, or a combination of both that occur during development. Cerebellar abnormalities range from subtle impairments including cognitive impairments to significant structural defects with life-threatening or lifelong disabilities [6].

Cerebellar dysfunction that disturbs the regulation of muscle tone, motor control, and coordination of movement is called ataxia – a broad term that refers to a disturbance in the smooth performance of the motor activities. The nonmotor dysfunction that results from cerebellar manifestations includes cognitive affective syndrome that includes impairment in executive function, spatial cognition, personality changes, and language deficits [7–9]. 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 the formation of cerebellum, clinical classification of cerebellar neurodevelopmental disorder is difficult; however, there are classification that are based on embryological and genetic considerations [16, 17]. Before the introduction of MRI, Dandy–Walker variants was a term used to characterize several types of cerebellar malformations. Now, cerebellar malformations can be classified into primary (malformation) and secondary (disruptive) lesions [17].

Secondary disruptive cerebellar defects are secondary to a developmental disorder in structures around the cerebellum such as Chiari malformation and 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, which are classified into four types (I–IV), with type IV being the most severe malformations [18]. Vein of Galen

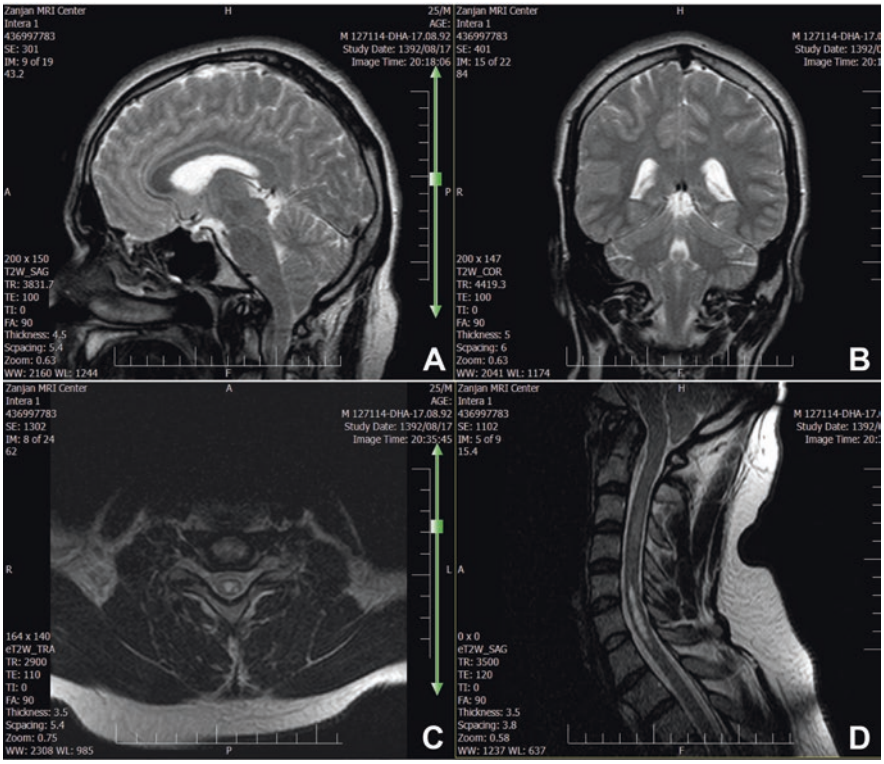

Fig. 1 Chiari malformation type I. (**a**, **b**) Sagittal and coronal T2-weighted brain MR images. There is slight inferior herniation of cerebellar tonsils through the foramen magnum that is less than 5 mm and shows benign tonsillar ectopia that could be a mild variant of Chiari malformation. (**c**, **d**) Axial and sagittal T2-weighted MR images of the brainstem and cervical spinal cord. Note the presence of a large syrinx in association with tonsillar ectopia in Chiari malformation type I

malformation is another secondary cerebellar 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 [19, 20]. In patients with vein of Galen malformation, the superior cerebellar arteries also discharge into the vein of Galen [21]. 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 [22], leading to cerebellar signs and symptoms.

Primary cerebellar malformations are classified into two broad categories: (1) those with hypoplasia and (2) those with dysplasia. Both hypoplasia and dysplasia categories have their own subgroups, which are categorized in Diagram 1 [17]. This chapter aims to discuss primary cerebellar malformations and the current treatment approaches in affected patients. The included primary cerebellar malformations are the cerebellar hypoplasia (CH), cerebellar dysplasia (CD), Dandy–Walker malformation (DWM), pontocerebellar hypoplasia (PCH), Joubert syndrome and related

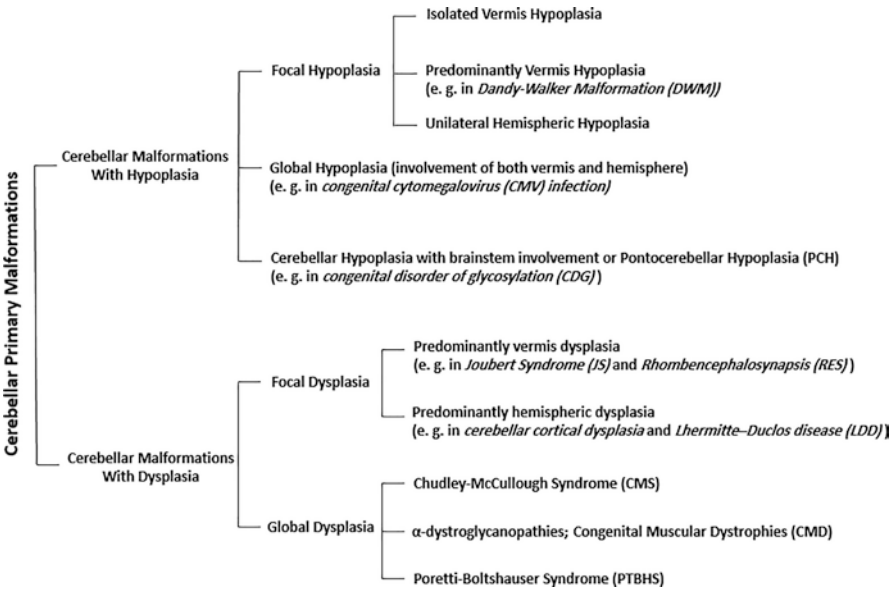

Diagram 1 Classification of cerebellar primary malformations

disorders (JSRDs), rhombencephalosynapsis (RES), lissencephaly with cerebellar hypoplasia (LCH), and dysplastic cerebellar gangliocytoma or Lhermitte–Duclos disease (LDD).

Cerebellar Hypoplasia

Cerebellar hypoplasia (CH) is a heterogeneous group of disorders that was first reported by Crouzon in 1929. From the embryological aspect, the cerebellar primordium emerges at approximately 28 days after fertilization in human (embryonic days 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) [6, 23–25]. 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 [26–28].

Distinguishing CH from cerebellar atrophy is very important because the treatment approaches are different. CH refers to a cerebellum with reduced volume, but with normal shape, which is stable over time and normal interfoliate fissures and sulci. On the other hand, cerebellar atrophy represents a progressive loss of cerebellar parenchyma along with secondary enlargement of both interfolial spaces and the fourth ventricle (29).

The causes of the CH are broad and include chromosomal aberrations (such as trisomy 9, 13, and 18), metabolic disorders [29], teratogens (drugs and infections: see chapter "Hormonal Regulation of Cerebellar Development and Its Disorders"), or isolated genetic CH (such as very low density lipoprotein receptor (VLDLR) – a reelin receptor [30, 31], dyskerin pseudouridine synthase 1 (DKC1) [32], oligophrenin 1 (OPHN1) [33], pancreas-specific transcription factor 1a (PTF1A) [34], and carbohydrate-deficient glycoprotein syndrome Types I and II (CDG 1 and 2) [35, 36]). Mutations in CHD7 (chromodomain helicase DNA binding protein 7) and retinoic acid exposure during early pregnancy are associated with variable cerebellar CH [31]. Similar to most developmental anomalies, CH may be associated with other brain malformations and there may be multi-organ involvement. Based on recent studies, CH is classified into three categories: (1) focal hypoplasia which has three subgroups – (i) isolated vermis hypoplasia, which is mostly characterized by partial absence of the inferior portion of the vermis, (ii) predominantly vermis hypoplasia including DWM [37], and (iii) unilateral hemispheric hypoplasia; (2) global (diffuse or generalized) hypoplasia (involvement of both vermis and hemisphere), which is more common in congenital cytomegalovirus (CMV) infection; and (3) CH with brainstem involvement including PCH [17, 38–40].

Clinically, in cerebellar hypoplasia, ataxia and poor motor learning are the most common and nonprogressive presentations compared with atrophic cerebellar disorders [41]. 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 [42].

Management It is important to consider that ataxia or other neurological signs in cerebellar hypoplasic patients usually do not worsen over time compared with atrophic cerebellar disorder. There is no standard course of treatment; therefore, the principal treatment is supportive including physical therapy, occupational therapy, speech therapy, psychiatric/behavioral medications, and special education (see chapters "Clinical Features, Assessment, and Management of Patients with Developmental and Other Cerebellar Disorders" and "Rehabilitation in Cerebellar Ataxia").

Cerebellar Dysplasia

Cerebellar dysplasia (CD) is defined by abnormal pattern in foliation**,** abnormal white matter arborization, heterotopic nodules of gray matter, and abnormal gray-white matter junction. CD may be associated with cysts resulting from disorganized cortical structures and pia matter disruption in which subarachnoid space is abnormally engulfed by dysplastic cerebellar folia [43]. CD can be subdivided into: (1) global CD, which has been reported in some posterior fossa malformations such as Chudley-McCullough syndrome, α-dystroglycanopathies, *GPR56*-related

polymicrogyria, and Poretti-Boltshauser syndrome; and (2) focal CD including dysplasia of the superior cerebellar vermis in Joubert syndrome and increased volume of multiple cerebellar folia in Lhermitte-Duclos disease [43]. In addition, it has been reported a rare number of isolated unilateral cerebellar hemispheric dysplasia [44, 45].

It has been reported that CD with abnormal folia orientation may be a core finding in PROS (PIK3CA-related overgrowth spectrum) patients that show somatic mutations in PIK3CA pathway genes, especially in those presenting MCAP (megalencephaly-capillary malformation) condition. Brain MRI of PROS patients, who are presented in clinics with vascular anomalies (mainly capillary malformations), segmental overgrowth dysregulation, and distal limb anomalies such as syndactyly and polydactyly, should be focused on cerebellum to detect any cerebellar dysplasia, which could be followed by proper genetic testing [46].

Management Like CH, treatment of CD is symptomatic and supportive.

Dandy–Walker Malformation

The fundamental structure that is affected in Dandy–Walker malformation (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]. Deletion of *Zinc finger 1* and *4* (ZIC1, ZIC4) genes on chromosome 3q24 [37, 50] and the *Forkhead Box 1* (FOXC1) gene on chromosome 6p25 are candidates involving in DWM [37, 51]. It is suggested that ZIC1 and ZIC4 are required for the full responsiveness of granule cell precursors (GCPs) to sonic hedgehog (SHH) [28]. It seems that FOXC1 directly regulates the size of posterior fossa and FOXC1-dependent SDF1α-CXCR4 (stromal cell derived factor 1α–CXC motif chemokine receptor 4) signaling from the surrounding mesenchyme to the developing cerebellar anlage regulates a plethora of cerebellar developmental programs [37]. Some other congenital abnormalities, especially eye malformations consistent with Axenfeld-Rieger syndrome, are seen in FOXC1-related DWM, and sometimes overlap with 3C (cranio-cerebello-cardiac) syndrome in severely affected patients [31]. Recently discovered mutations in CCDC22 (coiled-coil domain containing 22) gene in X-linked cases of 3C syndrome indicate that the CCDC22 mutations may be another cause of DWM [31]. Deletion of FOXC1 can lead to vermian tail (a common extended and dysplastic posterior vermis with an indistinct choroid plexus) in infants of DWM [52]. Autosomal dominant mutations in LAMC1 (laminin subunit gamma 1) and NID1 (nidogen 1) can also be considered as another causes of DWM [31].

DWM is characterized by agenesis or hypoplasia of the cerebellar vermis, upwardly rotated vermis, cystic dilatation of the fourth ventricle into the posterior cranial fossa, and an enlarged posterior cranial fossa [1, 37, 38, 53]. Enlargement of the posterior cranial fossa causes an abnormally high tentorium above the internal occipital protuberance and transverse occipital sulcus (location of transverse sinus),

as well as 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 hydrocephalus 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 global developmental delay (GDD), language delay, intellectual disability (ID), hypotonia, motor delay, 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 brainstem and nerves and can cause difficulties in controlling face and neck, and abnormal breathing patterns. Sagittal and axial MR images (Fig. 2) can distinguish DWM from other cerebellar malformations. In DWM, it is important to consider mega cisterna magna, retrocerebellar cysts, and Blake's pouch cyst [55, 59]. It should be noted that in addition to the absence of the middle part of the cerebellum, midline structures in the forebrain such as the corpus callosum may be absent, a condition known as agenesis of corpus callosum (ACC). 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].

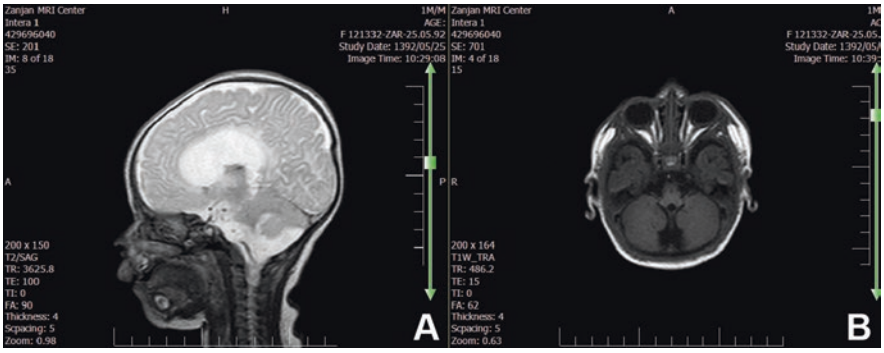

Fig. 2 Dandy–Walker malformation. (**a**) Sagittal T2-weighted brain MR image showing hypoplasia of the inferior vermis. A connection between the cisterna magna and the fourth ventricle is seen. (**b**) Axial T1-weighted brain MR image showing a cerebellum with isolated inferior vermian hypoplasia and normal cerebellar hemispheres, which is referred to as part of the Dandy–Walker variant

Management If there is hydrocephalous, treatment could include shunting and CSF drainage from the lateral ventricles and/or posterior fossa cyst, which is currently considered the ordinary surgical treatment of choice [49, 64]. Another way of shunting is endoscopic method, including (1) endoscopic third ventriculostomy (ETV), (2) aqueduct stent with shunt insertion, and (3) trans-tentorial proximal catheter insertion with endoscopic shunting [64]. The treatment 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 [65]. It is important that the families of affected children be referred for genetic counseling.

Joubert Syndrome and Related Disorders

Joubert syndrome (JS) was first identified by Marie Joubert in Montreal, Canada [66]. 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 [67, 68]. Molar tooth sign (MTS) observed on axial images of plain MRI (Fig. 3) is one of the gold standards of JS, which is formed by cerebellar vermis hypoplasia and dysplasia (most likely with a cleft in the superior vermis) accompanied by long, thick, elevated, and horizontally oriented superior cerebellar peduncles, with a deep interpeduncular fossa at the level of a thin midbrain–hindbrain junction (isthmus) [17, 37, 69]. In addition, diffusion tensor imaging (DTI), an MRI technique for white matter tractography, can further demonstrate laterally displaced and dysmorphic cerebellar nuclei, hypoplastic medial lemnisci, absent transverse fibers in vermis, and deficient superior cerebellar peduncle decussation [31].

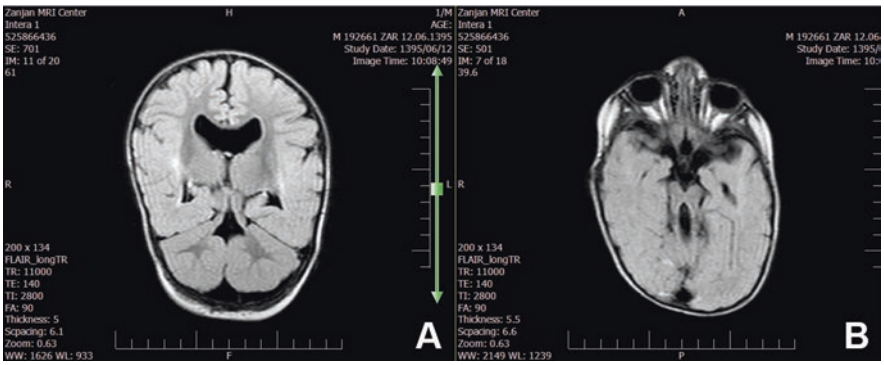

Fig. 3 Joubert syndrome and related disorders. (**a**) Coronal T2 FLAIR brain image. The cerebellar vermis is aplastic and superior cerebellar peduncles are elongated. (**b**) Axial T2 FLAIR brain image. This image shows a deep interpeduncular fossa, elongated superior cerebellar peduncles with cerebellar vermis hypoplasia, which are characteristic of the molar tooth sign in Joubert syndrome

When other organs, such as the retina, kidney, and liver are involved, it is called Joubert syndrome and related disorders (JSRDs), and these patients also have the MTS [67].

JSRDs is the most common inherited congenital cerebellar malformation in which ciliopathy is a fundamental mechanism. The primary cilia are important in neuronal development and function as cellular antenna that are found in nearly all cell types. Two main conditions related to defective function of primary cilia are retinal dystrophy and nephronophthisis that are found in many patients with JS [70]. 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 [71]. 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 [72, 73].

The causative gene of many ciliopathies in individuals with JSRDs has defined a new class of neurological diseases [71]. To date, over 16 causative genes have been associated with JSRDs and all encode proteins in the primary cilium or its apparatus [67]. For example, mutations in genes such as AHII, INPP5E, CC2D2A, and ARL13B cause JS with MTS and retinal blindness [74]. 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 and complete situs inversus [70], while mutations in TMEM67 are the most common cause of MTS with liver involvement [75].

Clinically, JSRDs patients have developmental delay, motor disability, hypotonia, ataxia, dysregulated breathing rhythms such as apnea and tachypnea (that results from dysfunction of the respiratory centers in the brainstem or cerebellum [69, 76]), abnormal eye and tongue movements, and subsequent mental retardation [70]. As 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) [77–79]. In mild JSRDs, ataxic movement lessens with age and the ability to walk is delayed to age 4–5 years.

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 because some neonates have died as a result of apnea. In this case, caffeine may be helpful to promote respiratory drive. These patients should be periodically examined for any non-neurological signs and symptoms. 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 retina, kidney, and liver [6, 80].

Pontocerebellar Hypoplasia

Pontocerebellar hypoplasia (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 [17, 38, 81, 82].

Ten different subtypes have been reported based on clinical and genetic features (i.e., PCH1–10) [83], 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 non-neuronal cells. Mutations in the vaccinia-related kinase 1 (VRK1) gene on chromosome 14q32.2 cause PCH1A (or spinal muscular atrophy with pontocerebellar hypoplasia; SMA-PCH), in which there is spinal cord anterior horn cell degeneration [84, 85]. Mutations in the EXOSC3 (exosome component 3) gene on chromosome 9p13.2 lead to PCH1B [86]. Mutations in three genes, TSEN54, TSEN34, and TSEN2, encoding three of four subunits of the tRNA splicing endonuclease (TSEN) complex have been found to underlie PCH2, PCH4, and PCH5 [81]. PCH2 is characterized by CH in which the hemispheres are more severely affected than the vermis, and in contrast to PCH1, 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, seizures [81, 82], early hyperreflexia, developmental delay, and feeding problems [31]. In brief, it is known that mutations in TSEN54 on chromosome

Table 1 Types of PCH

Gene	Chromosome	PCH types
VRK1	14q32.2	PCH1A
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

17q25.1 cause PCH2A; mutations in TSEN2 on chromosome 3p25.2 cause PCH2B; mutations in TSEN34 on chromosome 19q13.42 cause PCH2C; mutations in SEPSECS (*O*-phosphoseryl-tRNA:selenocysteinyl-tRNA synthase) on chromosome 4p15.2 cause PCH2D (as known as progressive cerebello-cerebral atrophy; PCCA); mutations in the gene VPS53, a subunit of the Golgi-associated retrograde protein (GARP) complexes, on chromosome 17p13.3 cause PCH2E; and mutations in TSEN15 on chromosome 1q25 cause PCH2F [87–89]. PCH3 that seems to be associated with optic atrophy is caused by mutations in the gene encoding PCLO (piccolo presynaptic cytomatrix protein) on chromosome 7q21 [90, 91]. PCH4 is caused by a mutation in the TSEN54 gene on chromosome 17q25.1 [87]. PCH4 is associated with polyhydramnios, contractures, severe hyperreflexia, and early death because of central respiratory failure [31]. A mutation in the TSEN54 gene on chromosome 17q25 causes PCH5, and mutations in the RARS2 (mitochondrial arginyl-tRNA synthetase 2) encoding gene on chromosome 6q15 cause PCH6, which is associated with elevated CSF lactate level [92]. The gene involved in PCH7 is unknown [93, 94]. PCH8 is caused by recessive loss-of-function mutations in the CHMP1A (charged multivesicular body protein 1A) encoding gene on chromosome 16q24 [95]. Mutations in the AMPD2 (adenosine monophosphate deaminase 2) gene on chromosome 1p13 cause PCH9, which is associated with severely delayed psychomotor involvement, progressive microcephaly, spasticity, and seizures [31, 96], and mutations in CLP1 (cleavage factor polyribonucleotide kinase subunit 1) gene on chromosome 11p12 cause PCH10, which is associated with progressive neurodegenerative features or static encephalopathy [31, 97]. Finally, loss-of-function mutations in SLC25A46 (solute carrier family 25 member 46) cause lethal congenital PCH [98].

Disorders presenting with PCH are constantly growing. Some examples are calcium/calmodulin-dependent serine protein kinase (CASK)-related PCH (associated with progressive microcephaly, hypoplasia of pons and cerebellum, intellectual disability, and epilepsy in female), congenital disorders of glycosylation (CDG; associated with GDD, language delay, eye anomalies, coagulation defects, neuropathy, impaired liver function, abnormal fat distribution, and cerebellar and pons atrophy imitating PCH), cerebellofaciodental syndrome (BRF1-related PCH; associated with microcephaly, short stature, intellectual disability, cerebellar and brainstem hypoplasia, and dystrophic features including taurodontism), and osteogenesis imperfecta (WNT1-related PCH; associated with developmental defects of the midbrain, pons, and cerebellum including variable degree of cerebellar and brainstem hypoplasia) [17, 38].

Clinically, PCH patients have hypotonia and difficulty with coordination of sucking and swallowing, and problems with handling their oral and respiratory secretions [99]. 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. Flattened cerebellar hemispheres (the “wings”) and a slightly preserved vermis (the

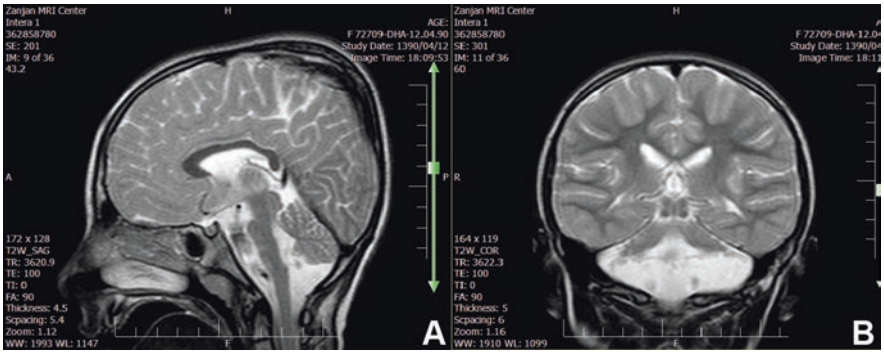

Fig. 4 Pontocerebellar hypoplasia. (**a**) Sagittal T2-weighted brain MR images. The pons is very small but has a relative sparring bulging in its superior part. Vermis hypoplasia predominates at the inferior site. (**b**) Coronal T2-weighted brain MR images. Cerebellar hemispheric hypoplasia with vermis relatively spared leading to classic dragonfly image

"body") together create dragonfly appearance on coronal MRI of PCH patients [17] (Fig. 4).

Management There is no known cure for PCH, and treatment is symptomatic and palliative 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. It is important to refer families of affected children for genetic counseling.

Rhombencephalosynapsis

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 [100, 101]. RES is a 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, a fused superior cerebellar peduncle, and midline dentate nucleus, creating a horseshoe-shaped arch across the midline [17, 37, 100]. Coronal T2-weighted MR images show horizontal folia pattern and mid-sagittal T1-weighted MR images show the dentate nucleus [17].

RES may be associated with other cerebellar abnormalities, such as Purkinje cell heterotopias [102]. Although RES is seen most frequently in isolated form, it can also be seen together with other developmental malformations in the nervous system or other organs. RES is a highly consistent finding in Gomez–Lopez–Hernandez syndrome (GLHS), which is also known as cerebellotrigeminal-dermal dysplasia (a

neurocutaneous disorder) presenting with parietal/temporal alopecia (focal dermal dysplasia or 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 [100]. RES has also been reported in VACTERL (vertebral anomalies, anal atresia, cardiovascular anomalies, trachea–esophageal fistula, renal anomalies, limb defects) association and hydrocephalus [42, 102–105].

Ishak et al. (2012) 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 [100]. 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 association 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). Based on other literatures, RES includes some specific clinical phenotypes such as biparietal alopecia, craniosynostosis, trigeminal anesthesia, and atresia of the fourth ventricle [106].

Clinically, signs and symptoms in patients with the isolated form of RES are variable such as developmental delay, in which motor learning and skills develop between 3 and 6 years of age, hypotonia, ataxia, abnormal eye movements, and head stereotypies [17, 107].

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 with Cerebellar Hypoplasia

Lissencephaly with CH 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 [108–111]. 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 [112] that is involved in migration of neurons through binding to its receptors (VLDLR), the

apolipoprotein E receptor 2 (ApoER2) [113–115], and also α3β1 integrin and protocadherins [116]. 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 [117, 118]. Mutations in RELN also show abnormal developmental disorders outside the brain such as neuromuscular connectivity and congenital lymphedema [110]. It is also reported that mutations in α-dystroglycan may result in lissencephaly and central nervous system developmental malformations [119].

Clinically, the important approach to diagnose is MRI of the cerebellum, which shows severe vermis and CH and cerebellar peduncle malformation.

Management Treatment of patients who have lissencephaly with CH are supportive care and symptom management. 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 Lhermitte–Duclos disease (LDD) was reported by Lhermitte and Duclos in 1920 as a cerebellar ganglion cell tumor or dysplastic cerebellar gangliocytoma [120, 121]. 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 [121–123]. Most frequently, LDD occurs in young adults in the third and fourth decades of life [124, 125]. 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. Long-standing unilateral space-occupying skull lesions in the posterior fossa leads to thinning of the skull in the occipital region [126, 127]. 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 [128].

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 [126]. LDD patients may show mental retardation. LLD is commonly associated with other disorders of cortical formation, megalencephaly, gray matter heterotopia, polymicrogyria, polydactyly,

macroglossia, localized gigantism, and 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 [17, 129]. Widened cerebellar folia with striated appearance can be seen on T2-weighted MRI [17]. Elevated lactate, slightly reduced *N*-acetyl aspartate (NAA), reduced myoinositol, reduced choline, and reduced choline/creatine ratio are common on MR spectroscopy (MRS) [17].

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, cerebellar malformations and current treatment approaches were summarized. Based on available knowledge and our clinical experience, there is no curative treatment and most of the patients are managed using conservative approaches (see chapters "Clinical Features, Assessment, and Management of Patients with Developmental and Other Cerebellar Disorders" and "Rehabilitation in Cerebellar Ataxia"). Treatment is in response to symptoms and requires a team of specialists (neonatologists, pediatricians, neurologists, and therapists), health-care professionals, and genetic counselors.

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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, social, and cognitive functions. Cerebellar disorders cause tremor and incoordination, increased variability and inaccuracy of movements during eye and limbs movements, stance, and speech. Cerebellar dysfunction also results in impaired cognition and behavior. 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, should be elicited during the clinical assessment. During examination, emphasis is placed on examining the motor system, especially speech, eyes, and limb movements. Other aspects include general examination, head size, dysmorphic features, neurocutaneous stigmata, and cognitive function assessment. A thorough examination of 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 and social 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 for 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]. 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 are available [8–11]. More recently, a consensus paper on the cerebellum and social cognition has been published [12].

One of the themes that may underlie the motor and nonmotor manifestations of cerebellar disease is the disturbance or loss of precise timing in a variety of tasks in which the cerebellum plays a central role [13].

Limbs Motor Control

Smooth and accurate execution of voluntary movements and adaptation to changing demands of motor tasks rely on an intact cerebellum [14]. 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 [15]. Cerebellar dysfunction causes greater impairment in predictive movements than in movements requiring feedback, for example, visual or somatosensory feedback [16]. Patients with cerebellar disorders appear to have proprioceptive deficits during active but not passive limb movements [17]. Furthermore, the ability to adapt to novel changes in movements is impaired. Table 1 shows several clinical motor signs in patients with cerebellar disease.

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 examiners 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 examination or heel-to-shin examination. 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 examination or heel-to-shin examination
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., 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 dysmetria
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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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 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 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 ocular motor vermis/fastigial ocular motor region [8, 18].

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, 18]. Saccadic (jerky) smooth ocular pursuit and saccadic dysmetria (hypo- or hypermetria) are other well-recognized ocular motor signs of cerebellar dysfunction [18]. 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 many muscles, in particular the tongue and orofacial muscles [19]. 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 [20].

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 [20]. 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].

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 upgaze
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 eyes 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 (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
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

(continued)

Table 2 (continued)

Sign	Comment
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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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 [21], with anomia, perseveration, and reduced speech output and speed [22, 23].

Cognition

Investigations on the cerebellar contribution to cognition are consistent with a role for the lateral cerebellar hemispheres in supporting cognitive processes [24]. In children, significant cognitive disruption is associated with pediatric cerebellar diseases ranging from cerebellar developmental abnormalities to inflammatory disorders, ischemic injury, and oncological and postsurgical injury [25–34]. 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. Furthermore, new evidence is emerging on the role of the cerebellum in social cognition both in social "mirroring" and

"mentalizing." Social impairments (e.g., the inability to understand the mental state of other people through nonverbal, usually visual, cues) seen in patients with degenerative ataxias and autism spectrum disorder have been postulated to occur as a result of cerebellar dysfunction [12].

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 [21]. 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 [21, 29, 35, 36].

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 [37]. 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, 38]. 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 [37], including headache, confusion, developmental regression, seizures, numbness, tingling, and weakness.

Age of the parents at conception, previous miscarriages, mother health and toxins 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 [38]. 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 [39–42].

Drug history and possible exposure to toxins or drugs should be obtained [4, 37]. 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 [43]:

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 may not be manifested 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, 37, 41, 42], 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 examination 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 [44]. A practical and comprehensive guide on the examination and interpretation of eye movements in children is available for the interested

reader [45]. 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) [18].

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 while taking history. In addition, watching the child play, use an iPAD, tablet, smart phone, 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 (Table 4)?

Extra-cerebellar features should be looked for to identify red flags [4, 37]. For example, swollen optic discs 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 such as

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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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 (e.g., irritable hip), and psychogenic disorders [37, 46].

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 explored using latent class analysis. Few specific clinical patterns emerged that were highly associated with certain disease etiologies [47]. 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 magnetic resonance imaging (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, for example, the molar tooth sign. Another example is a child who has no history of seizures, has symptoms onset including ataxia at greater than 10 years of age, and has 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 help 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 are 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, 18, 48].
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 [43]. 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, 37, 38, 43].
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 imply widespread central nervous system involvement beyond posterior fossa structures [43].
7. Are other organs affected? For example, heart, liver, or kidneys involvement raises suspicion of a metabolic disorder.

Based on the clinical impression, a plan of investigation is carried out [3, 37, 43, 47, 49]. Neuroimaging is usually very helpful, even when it is normal [46]. A brain MRI with magnetic resonance angiography and spectroscopy if indicated, offers the best spatial resolution of cerebellar and extra-cerebellar brain structures [46, 50]. A spinal MRI is occasionally helpful. For example, it may reveal spinal cord atrophy in patients with Friedreich ataxia [46]. 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 [46].

Biochemical tests, drugs and toxin screens, and metabolic investigations on blood, urine, and, where appropriate, cerebrospinal fluid are then performed in a stepwise manner [3, 5, 43]. 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 focusing, 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 [38, 50] and are usually also requested in a stepwise manner guided by findings from the clinical assessment and neuroimaging findings. The tests include microarray-based comparative genomic hybridization, karyotype, FISH, calcium channel mutations, and mutations in selected spinocerebellar ataxia genes. Ataxia gene panel testing is another option for diseases with similar phenotypes. Whole exome sequencing has become more widely available in routine clinical practice. It is proving to be a useful investigation in patients with undiagnosed ataxia.

Nerve conduction studies, electromyogram, electroencephalogram, evoked potentials of the visual (VEP), auditory (BAEP), and somatosensory (SEP) systems may also be indicated in some patients with ataxia, who have additional clinical features, e.g., peripheral neuropathy or seizures. Abnormalities found in these investigations are reflective of the widespread pathology in many subtypes of genetic or hereditary ataxias [49]. Skin and muscle biopsies are being done less often nowadays since genetic testing has become more widely available.

Formal qualitative and quantitative assessment of speech, gait, and eye movements are available in large medical centers. They rarely contribute to diagnosis. They are more useful in assessing response to therapy, especially in clinical trials [49].

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 [51]. 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].

There is some evidence that the cerebellum can compensate for: (1) The loss of its parenchyma when it is acutely damaged, e.g., by a stroke, and (2) loss of its function, e.g., in immune-mediated ataxias, through mechanisms involving neighboring cerebellar or extra-cerebellar regions in the former and the functionally impaired region itself in the latter. This cerebellar reserve has a limited time window during which every effort should be made to stop further damage or eliminate/slow down the disease process; while at the same time, enhancing recovery through therapies that enable the cerebellum to potentially compensate, at least partially, for its lost function [52].

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 [53]. Other noninvasive cerebellar stimulation techniques are being explored and preliminary studies show possible therapeutic benefit [53, 54]. A recent consensus paper on neurostimulation of the cerebellum is available for the interested reader [55]. The paper

discusses invasive and noninvasive methods to stimulate the cerebellum to treat cerebellar ataxia and other neurological disorders including stroke and dystonia.

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 organization such as the National Ataxia Foundation can be invaluable to the patients and their families [43].

There are limited treatment options available for the ataxic patients [51, 54]. Treatments for developmental cerebellar disorders and most hereditary ataxias are generally not available. A systematic review in 2018 reported that for patients with ataxia of various etiologies including Friedreich's ataxia and spinocerebellar ataxias, riluzole is probably effective for short-term treatment of ataxia [54].

Specific treatments are only available for a handful of diseases that are usually caused by metabolic dysfunction [3, 5, 53]. For example, vitamin E is given to patients with abetalipoproteinemia or ataxia with vitamin E deficiency, biotin to patients with biotinidase deficiency, coenzyme Q10 to patients with coenzyme Q10 deficiency, acetazolamide or 4-aminopyridine to patients with episodic ataxia type 2 [54], 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 ketogenic diet to patients with pyruvate dehydrogenase deficiency or glucose transporter 1 (Glut-1) deficiency syndrome.

Other symptoms associated with ataxia should also be addressed and treated, e.g., epilepsy, spasticity, sleep disturbance, behavioral difficulties, and anxiety.

Patients with multisystem disease should be referred to other specialists [43]. 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 Q10.

Finally, the Covid-19 pandemic has impacted the care of patients with ataxia and complicated their management including attendance to clinic, speech and language therapy, and other rehabilitation services. In addition, ataxic patients may be at higher risk of being infected with the virus because of the spectrum of neurological and comorbid illnesses that accompany their diagnosis, e.g., cardiomyopathy and diabetes in Friedreich ataxia. The pandemic has also affected the mental and physical well-being of many of us given future uncertainty and anxiety, which in turn exacerbated the neuropsychological morbidity of patients with cerebellar disorders [56].

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 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 (see chapter "Rehabilitation in Cerebellar Ataxia") and disease-specific treatments are available.

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Conflict of Interest None.

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Rehabilitation in Cerebellar Ataxia

Jennifer L. Millar and Meredith P. Drake

Abstract The cerebellum is a unique structure that is densely connected to both motor and nonmotor regions of the brain and plays a critical role in coordinating and adapting movements. The most debilitating effect of damage to the cerebellum is resultant ataxia. Ataxia, derived from the Greek word meaning "lack of order," is a nonspecific term that refers to uncoordinated movements. Ataxia may also be used as a medical diagnosis. In this chapter, we will focus on this hallmark feature of cerebellar damage, which is incoordination of movements without overt muscle weakness, and we will discuss the potential benefits of rehabilitation and the importance of optimizing sensorial and motor experiences to promote motor learning.

Keywords Rehabilitation · Motor learning · Ataxia

Introduction

Cerebellar ataxias contribute to dysfunction of neurological pathways, influencing voluntary movements. Ataxias may be progressive or acquired in nature. Common symptoms include difficulties with balance, coordination, ocular motor, speech, and swallowing functions [1]. Evidence in the literature has shown that intensive rehabilitation is effective in managing motor limitations for optimizing function in people living with ataxia [2–9].

In this chapter we will highlight current rehabilitation literature in ataxia, principles behind the efficacy of rehabilitation, as well as evaluation and treatment considerations, including exercise and safe mobility strategies for symptom management and fall prevention.

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Motor Learning Principles

In healthy individuals, the role of the cerebellum is to optimize movement coordination as well as adaptation of new movement patterns to achieve a desired motor outcome [10]. It has been theorized that the cerebellum estimates a certain state of movement, and then forms a forward model predicting the sensory consequences of a movement [11–16]. If there is a discrepancy between the expected movement and actual movement, the cerebellum updates the forward model using a prediction error that is fed to the cerebellum by the parietal cortex [13]. Hence, motor commands are linked with the predicted sensory consequences of movement [15].

Examples of when the body needs to adapt to a new movement pattern in real-life circumstances includes finding an app on one's phone in after the app has been inadvertently moved, or driving a rental car that is different than your own vehicle, or adjusting to a Mac computer if you are more familiar with using a PC.

In healthy individuals, implementing appropriate movements and learning new movements are possible with the influence of sensory feedback from various systems (vision, somatosensory, vestibular). Visual feedback, in combination with vestibular feedback, contributes to stable gaze in response to head and body turns. Additionally, vestibular feedback, including linear and rotational accelerations, provides information of spatial orientation. Somatosensory feedback provides necessary information to accomplish precise movement at multiple joints. Importantly, the brain does not simply react to multimodal sensory feedback, but putatively uses an internal model of the body to predict the consequences of motor commands before sensory feedback arrives [15].

In individuals with cerebellar ataxia, the ability to adapt to new motor learning patterns, relative to previous experiences, is impaired [12]. However, reinforcement feedback learning has been established as an alternative motor learning strategy in cerebellar ataxia, where actions leading to a successful outcome are reinforced while unsuccessful actions are avoided [16]. Of interest, reinforcement motor learning has been shown to require a certain level of motor exploration to optimize and reduce variability of movement [16, 17].

Evidence in the Literature

Current evidence in the ataxia rehabilitation literature emphasizes the value of intensive balance training and aerobic exercise to improve and maintain clinical and functional outcome measures [18]. High intensity, coordinated balance training has been shown to improve clinical neurological scores and gait kinematic measures in adults and children with neurodegenerative ataxias, such as decreased variability of steps, body sway, and increased gait velocity [3, 7, 19]. While clinically meaningful improvements may be experienced in the short term, maintaining an intensive home program for the long term is essential for retaining motor improvements in ataxia [2].

A consensus as to the appropriate dosing has not yet been reached in the ataxia literature given the heterogeneity of individuals with ataxia. However, evidence shows that intensity of training (i.e., self-perceived level of difficulty) matters more so than frequency [7]. Intensive multidisciplinary inpatient rehabilitation has also been shown to improve functional outcomes in ataxia [4, 6]. Larger systematic reviews reveal consistent evidence that balance and endurance training, as well as multidisciplinary care has the potential to improve clinical and activity measures [5, 8, 9].

Evidence in the literature is primarily among the neurodegenerative, hereditary ataxias and in individuals who are ambulatory. Considering ataxia etiologies and level of severities are varied, future research is needed in larger patient cohorts to address questions such as dosage and motor learning preferences to optimize treatment.

Evaluation

Neurorehabilitation specialists often refer to a clinical framework called the International Classification of Functioning (ICF) model to guide the evaluation and overall management of impairments that may potentially restrict a person's overall abilities and participation in life activities [20]. Additionally, the ICF model helps organize which activity limitations are modifiable within the scope of rehabilitation. The ICF model also considers environmental factors, such as home setup, as well as personal factors, such as motivation and social support. It is important to consider that there are many factors that contribute to an individual's overall function and quality of life, beyond the medical diagnosis. Clinicians must assess how a range of factors are contributing to the patient's current functional situation, and work with the individual to address the limitations and barriers to life activities most meaningful to them.

Common motor impairments observed on the evaluation may include ocular motor abnormalities, limb dysmetria – impaired ability to reach a target accurately, dyssynergia – impaired ability to move smoothly and precisely at multiple joints simultaneously, and imbalance. Current rehabilitation literature focuses on management of motor impairments, impacting the person's overall activity, such as balance, gait, and endurance. However, it is important to acknowledge nonmotor impairments in ataxia, which may impact the patient's health-related quality of life. Recent murine evidence of cerebellar fastigial nucleus outputs involving both motor and nonmotor circuitry brings attention to nonmotor aspects of cerebellar disease including generalized arousal, motivation, wakefulness, and working memory [21].

Understanding ataxia etiology is important (i.e., hereditary neurodegenerative ataxia versus the consequence of a stroke, traumatic brain injury, or autoimmune event). However, an individuals' clinical presentation, as well as social, behavioral, environmental considerations, are equally important for rehabilitation management.

Examination

Key components of a physical therapy evaluation of an ataxia patient include an ocular motor exam; balance, coordination, sensation, strength, and gait testing; and functional and patient-reported outcome measures. The ocular motor exam should be performed in room light and with fixation removed (using goggles, such as Frenzel's lenses) and should include static and dynamic gaze, pursuits, saccades, as well as vestibular ocular reflex responses with passive head rotation. Common abnormal ocular motor and clinical findings are detailed in Table 1.

Table 1 Ocular motor and clinical exam

Ocular motor exam	**Abnormal findings**
Central gaze	Spontaneous nystagmus – downbeating, pendular, periodic alternating nystagmus
Lateral gaze	Direction changing gaze evoked nystagmus
Pursuits	Choppy, saccadic eye movements
Saccades	Latency, velocity, accuracy impairments
VVOR – visual vestibular ocular reflex	Difficulty maintaining focused on a stationary target with passive slow head movements or rapid head impulse
VVOR cancellation	Choppy pursuits. Difficulty maintaining focus on a moving target as the eyes, head, target move around the head axis simultaneously
Vergence	Convergence or divergence insufficiency
Positional testing	Sustained nystagmus greater than 1 min Slower velocity, smaller amplitude nystagmus than typically observed with peripheral BPPV Pure upbeating or downbeating nystagmus; immediate onset Nystagmus in more than 1 position
Dynamic visual acuity (DVA)	Impaired with or without evidence of vestibular involvement [22]
Video head impulse test (vHIT)	Abnormal VOR gains may be present in some cases Eye movement patterns in response to passive head rotation may reveal the following in one or more semicircular canal: [23] Hypometric VOR with saccades Hypermetric VOR Anti-compensatory saccades Premature VOR deceleration
Clinical exam	**Abnormal findings**
Sensation	Standing balance testing, using the mCTSIB, may be more informative than great toe proprioception
Strength	Strength is typically normal
Coordination	Dysmetria Dysdiadochokinesia Dyssynergia
Balance	Difficulty maintaining Romberg stance with feet together or in tandem on firm or foam surfaces, with eyes open or closed
Gait	Wide-based, discontinuous steps, difficulty with tandem walking, walking with head and body turns

- *Dynamic visual acuity* (DVA) testing may be incorporated into the clinical exam and reveal abnormal results in individuals with cerebellar ataxia [22]. In ataxia individuals with additional vestibular clinical findings, the DVA severity is comparable to individuals with bilateral vestibulopathy [22].
- *Video head impulse* testing (vHIT) is an available clinical tool, beyond the standard ocular motor exam, in identifying impairment in eye and head coordination, with passive head rotations. In ataxia individuals with specific complaints of oscillopsia with head motion, symptom severity is comparable to those with bilateral vestibulopathy [23]. In ataxia individuals with oscillopsia, vestibular ocular reflex (VOR) gains may be impaired in at least one semicircular canal [24]. Interestingly, oscillopsia symptoms are inversely correlated with gait velocity ($r = -0.55$, $p < 0.05$), but not with VOR gains [24]. An example of a video head impulse test result in an individual whose clinical presentation included symptoms of oscillopsia, limb ataxia, and sensory impairments is shown in Fig. 1. In this individual's case, genetic testing confirmed a diagnosis of cerebellar ataxia with neuronopathy and vestibular areflexia syndrome (CANVAS).
- *Sensation*: Proprioception testing, whether the patient can identify if his/her toe is passively oriented up or down, is not informative enough to fully understand an individual's potential for joint kinesthetic awareness with postural stability challenges. A modified clinical sensory integration and balance test (mCTSIB) is a useful tool for identifying somatosensory impairments pertaining to function. The mCTSIB consists of four subtests, each timed for 30 s each: [1] stance with feet together, firm surface, eyes open; [2] stance with feet together, firm surface, eyes closed; [3] stance on foam, eyes open; and [4] stance on foam, eyes closed. If the individual does have sensory ataxia symptoms, it is difficult for clinicians to distinguish between peripheral neuropathy and neuronopathy (i.e., ganglionopathy). However, individuals with neuronopathies may also have cranial nerve deficits (except for hearing loss) and will be distinguishable from neuropathy with the help of nerve conduction studies.
- *Strength*: Typically, strength is not an issue in degenerative cerebellar ataxias, but often individual's with impaired motor control complain of "weakness." In individual's who are less active, or fearful of falling, secondary weakness may be

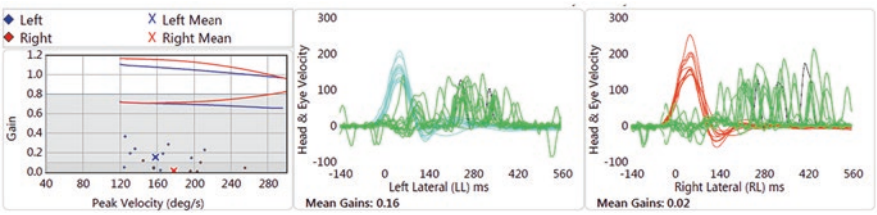


Fig. 1 On the left graph, the plots reflect the ratio of the area under the eye velocity curve relative to head velocity, otherwise known as visual vestibular ocular reflex (VVOR) gain. The normal VOR gain range would normally fall in between the red and blue lines on the left graph, or gain values of ~0.8–1.2. However, in this individual's case, VOR gains were impaired, with evidence of overt compensatory saccades bilaterally

more of an issue than primary neurological weakness, especially in hereditary ataxia conditions.

- *Coordination*: Evidence of dysmetria, dysdiadochokinesia, and dyssynergia may be evident with finger to nose movements, rapid alternating movements, and heel to shin movements. Clinical findings of incoordination may be subjectively rated using one of the validated ataxia rating scales described below.
- *Gait*: Common gait patterns in ataxias may include wide-based discontinuous steps, difficulty walking in tandem, with head and body turns, or with a dual task cognitive challenge.

Clinical Outcomes

The most common clinical ataxia rating scales used by neurologists and therapists are the International Cooperative Ataxia Rating Scale (ICARS) [24] and Scale for Assessment and Rating of Ataxia (SARA) [25].

The *International Cooperative Ataxia Rating Scale* (ICARS) [24] is a 19-item scale with posture, gait, limb ataxia, dysarthria, ocular motor subscales. Total score ranges from 0 to 100, with a higher score implying a greater impairment. The ICARS is considered to have high interrater reliability (ICC = 0.95) and high test–retest reliability (ICC = 0.97). [26, 27]

The *Scale for Assessment and Rating of Ataxia* (SARA) [25] is an 8-item scale with balance, gait, limb coordination, dysarthria subscales. Total score ranges from 0 to 40, with a higher score implying a greater impairment. The SARA is considered to have high interrater reliability (ICC = 0.98) and high test–retest reliability (ICC = 0.90) [26]. The EuroSCA natural history study revealed an average annual SARA score progression of up to 0.6–2.5 points per year among the most common hereditary degenerative spinocerebellar ataxias [28].

Functional Outcomes

The *Action Research Arm Test* (ARAT) is a 19-item measure to evaluate upper extremity function coordination, dexterity in neurological individual's [29]. The ARAT, in cerebellar ataxia, has high interrater reliability (ICC = 0.97) [30].

The *Dynamic Gait Index* (DGI) is an 8-item fall risk measure with various functional tasks, including head and body turns and stepping over and around objects [31]. A total score of less than 19/24 indicates risk for falls. The DGI, in cerebellar ataxia, has high interrater reliability (ICC = 0.98), high test–retest reliability (ICC = 0.98), and construct validity ($r = -0.81$ SARA, $r = -0.88$ ICARS) [32].

The *Timed Up and Go* (TUG) measures the duration to stand, walk 3 m, and turn 180° before returning to sit. The TUG indicates fall risk when scores are >13.5 s in

older adults with vestibular disorders. The TUG has high inter and intrarater reliability [33].

The TUG with a dual task challenge may include a cognitive task (TUG Cog), such as counting backwards from 100 by 3 s. A TUG Cog score of >15 s in elderly subjects has been used to identify individuals with increased fall risk [33]. The TUG Cog score is helpful when assessing how dependent an individual with ataxia is on cognition for motor performance.

Gait velocity measures comfortable gait speed and may be captured with or without an assistive device. Normal age range values are available in community dwelling adults and are not explicitly known for patients with ataxia [34].

The *Five Time Sit to Stand* test is a useful clinical measure of an individual's ability to transition from sit to stand five times sequentially. The measure has been validated in individuals with balance disorders [35].

Patient Reported Outcomes

Activities-Specific Balance Confidence scale (ABC) evaluates an individual's level of perceived balance confidence with various daily activities ranging from 0 (no confidence) to 100% (complete confidence). Total scores of >80% is considered a normal level of balance confidence and scores below 67% predict an individual is at risk of falling [36]. The ABC has excellent test–retest reliability ($r = 0.92$) [37, 38].

Oscillopsia Functional Index (OFI) was initially developed to assess oscillopsia symptoms in patients with peripheral vestibular dysfunction [39]. The 43-item questionnaire asks patients to rate their level of oscillopsia during various activities such as walking, driving a car, or the ability to recognize familiar faces. Scores range from 0 to 215 points, ranked from 0 (no oscillopsia symptoms) to 5 (severe oscillopsia such that the person has stopped doing the activity). A scoring option of "not applicable" reflects the people's avoidance of a particular activity. The OFI has high internal consistency with excellent validity and is correlated with other oscillopsia measures (oscillopsia visual analog scale: $r = 0.69$, $p < 0.001$; oscillopsia severity scale: $r = 0.84$, $p < 0.0001$). The OFI is also correlated with the Activities-Specific Balance Confidence scale ($r = -0.84$, $p < 0.001$) [38].

In summary, patient-reported outcomes, as well as clinical and functional outcome measures, are valuable tools for identifying self-perceived level of disability, clinical impairments, and functional limitations impacting daily activities. These measures also provide individuals with helpful feedback on the value of maintaining home exercise program compliance.

Rehabilitation Interventions

In general, functional recovery is possible in individuals with ataxia, and the extent of recovery depends on the cause and site of the cerebellar lesion. Degenerative cerebellar ataxias are difficult to treat due to the progressive nature and its effects on all parts of the cerebellum. In contrast, nondegenerative ataxias, such as poststroke, may affect only some regions of the cerebellum, leaving the intact regions available to assist with compensation. Rehabilitation of cerebellar-induced motor impairments is also complicated by the role of the cerebellum in motor learning. Poor functional recovery may be a consequence of damage to cerebellar structures involved in adaptation [18].

The literature for specific rehabilitative interventions for ataxia is limited. The few available studies feature different populations (e.g., degenerative ataxias, poststroke ataxia, sensory ataxia, postsurgical cerebellar tumor resection, multiple sclerosis), as well as different interventions and outcome measures. Thus, here we will highlight common rehabilitation therapies for cerebellar ataxia featured in the literature as well as practical clinical considerations based on current evidence.

It is important to note that ataxia symptom management is often in contrast to rehabilitation management strategies for other movement disorders. For example, Parkinson's disease management commonly focuses on high-amplitude and high-velocity movements, with external cueing strategies, whereas ataxia management focuses on slow, soft movements. Distinguishing the type of movement disorder, such as ataxia versus Parkinson's disease, is important for guiding treatment.

Intensive Balance and Gait Training

Many of the intervention studies for cerebellar ataxia emphasize stability and balance training [3–8]. Literature has shown high-intensity balance training may have indirect effects on quality of walking and gait velocity. Interestingly, balance deficits have been found to be a better predictor of gait speed than leg-coordination deficits in neurodegenerative ataxias [40]. Intensive coordinative training has been found to improve gait performance and reduce ataxia symptoms up to 2–5 SARA points. The natural disease progression of the most common degenerative cerebellar ataxias per year is 0.6–2.5 SARA points [28]. Therefore, neurodegenerative ataxia patients who participate in intensive balance training have the potential to gain clinical improvements equivalent to 2 or more years of disease progression [3]. Additionally, several studies, in individuals with neurodegenerative ataxias of varying disease severity, have shown kinematic improvements correlating well with improvements in functional and clinical neurological scores following intensive balance training [3, 7, 41]. Long-term improvements have been observed in individuals who continue their home program [2]. Functional improvements are more likely experienced among individuals with pure cerebellar ataxia, more so than those with sensory ataxia [3]. The self-perceived difficulty of a task is important for achieving benefit. Exercises must be safe, yet challenging [7].

Table 2 Example of balance and gait exercises

Bed / floor exercises: *All exercises are intended to improve core stability and coordination in preparation for standing tasks.*

A- Bridges are an effective exercise for strengthening the hips and core, and may be progressed by performing with arms across chest, or with a narrow base of support.

B- Progression: Bridges with reduced support (alternating heel raises)

C- Progression: Bridges with removed support (alternating leg raises).

D- Marching is a both a core stability and coordination exercise, with emphasis on slow, controlled, movements.

E- Stabilizing on half round roller without arm support, with knees apart.

F- Half foam roller progression: adding alternating arm and/or leg raises.

Foam roller size: 36" x 6" x 3"

Quadruped exercises:

G- Maintaining a quadruped position can be a challenging core stability task in itself.

H- Progression: lifting one arm or leg at a time, or simultaneously lifting one arm and the opposite leg.

General balance training recommendations:

Frequency/Duration:
4 times/week, 20 min minimum.

***Intensity**: Challenge matters! Yet, individuals are encouraged to modify tasks to ensure safety and success. Progressions should be gradual, with small incremental changes.*

Focus on quality of movement, not speed!

Dynamic sitting balance exercises: *(edge of chair)*

I- Marching in place: Stabilize your gaze on your hand as you reach across your body to tap your opposite knee. Switch.

J- Side to side weight shifts: Lean your body to the side while maintaining your gaze on your hand.
Hold 5 s. Return to center.
Switch.

K- Forward and backward weight shifts: Lean your body back with your hands in front. Avoid touching the back of the chair.
Then lean your body forward, with your hands back, head up. Repeat.

L-"V" sit hold: scoot forward in the chair so your back is not supported. Lean back without touching the back rest, and hover your feet off the ground. Try to remain balanced and hold 10 s.

M- Squats in standing:
This should be performed under the advice of a physical therapist and/or with a second person; as well as a chair behind and/or walker in front.

- Squat with your hips positioned back, feet apart, eyes open.
- Hold 5–10 s. Repeat.

Common balance interventions include exercises in various positions. Home exercises of sufficient difficulty may indirectly improve gait performance [7]. Examples of balance and gait exercises, as well as samples of progressions are highlighted in Table 2.

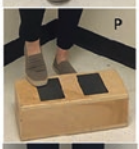
Locomotor training overground and on treadmills, both with and without body weight support, has been used with some success in limited case studies [42, 43]. It is important to note that utilizing body weight support can be useful for achieving intensity and repetition to facilitate plasticity of the brain; however, it should be considered that body weight support minimizes balance challenge.

Static standing balance exercises:
Standing tasks should be performed in a corner of a room for safety, with a chair or walker in front for an extra point of balance; or with a second person.

N- *Standing with narrow base of support or feet together:*
Arms across chest, or at side, eyes open.
Progression: closing eyes or turning head slowly.

O- Semi-tandem stance:
(standing with feet overlapped)
Progression: gradually decreasing the amount of overlap, 1 inch at a time.

P- *Step stance:*
(standing with one foot on a step or stool)
Progression: standing with feet positioned slightly closer; adding intermittent eye closure, or adding slow head turns.



Q- *Standing on a compliant surface:*
(pillow), feet apart, arms across chest.
Progression: standing with feet closer together or feet overlapped; adding intermittent eye closure, or head turns.

Dynamic standing balance and gait exercises:
These higher-level interventions should be performed under the advice of a physical therapist and/or with a second person.

Dynamic standing balance exercises:
- Marching in place, arms across chest, eyes open.
- Standing toe taps: forward, backward, to the side.
- Standing alternating toe taps onto a stool.
- Standing reaches: standing still while reaching one arm forward, backward, to the side.
- Squats, feet apart, eyes open.
- Standing body turn: looking over shoulder.
- Weight shifting: side to side.
- Weight shifting: front and back.

Gait exercises:
- Walking with narrow base of support, arms at sides.
- Walking with head turns (vertical and horizontal directions each).
- Walking with wide 180° turns.
- Walking with intermittent eye closure. (ie walking 3 steps eyes closed, 1 step eyes open, repeat)
- Multi-directional walking: sideways and backwards.
- Walking on inclines/declines.
- Walking semi-tandem (with feet overlapped).
- Walking around and over obstacles.

Table. 24.2 (continued)

A systematic review published in 2017 highlighted 17 studies, among 292 individuals with ataxia [8]. Treatment interventions included balance and coordinative training, multidisciplinary inpatient rehab, cycling, and treadmill training. Fifteen of the 17 studies revealed significant improvements in at least one outcome measure among ataxia symptoms, balance, function, and gait. The review revealed consistent evidence that rehabilitation improves function, mobility, ataxia, and balance in genetic degenerative ataxia. Interestingly, training intensity and frequency varied among studies. In some studies, individuals were asked to train 1 h per day, 7 days per week, while others just 20 min, 4 times per week, with a higher self-perceived level of challenge. The latter frequency/duration, with a higher intensity, has been shown to be the minimum dosage necessary to achieve significant improvement in gait velocity [7]. Rehabilitation professionals have a significant role in helping individuals establish the appropriate exercise dosage and type, based on response to training.

Aerobic Exercise Training

Integration of aerobic exercise into the regimen is recommended, given with ataxia, movements are more effortful, requiring increased energy expenditure. Fatiguing activity worsens postural control [44] and contributes to risk for falls. Interestingly, balance capacity correlates with exercise performance [45].

The severity of ataxia has been associated with poor physical conditioning, decreased overall functionality, and lower quality of life [45]. Health professionals

should promote physical exercise to help people with cerebellar dysfunction in preventing functional losses caused not only by disease but also by inactivity. One study found cycling can normalize the modulation of reciprocal inhibition, restore short- and long-term adaptive plasticity, and improve coordination function in the individuals with ataxia [46]. Another study involving a high-intensity cycling program found a 2-point reduction in severity of ataxia based on SARA scores, while the control counterparts increased by 0.3 points [9]. The results also revealed improvements in walking speed, balance outcomes, and general fitness. Thus, while it is well-established that balance and coordinative training are especially important in the rehabilitation of cerebellar dysfunction, cardiovascular and muscular fitness should not be neglected, and should be remembered as an important part of a routine exercise program for people with cerebellar dysfunction.

In individuals with Friedreich's ataxia, a more cautious approach to aerobic exercise is recommended. Rehabilitation has been shown to be effective in reducing the level of disability in Friedreich's ataxia [47, 48]. Given the prevalence of cardiomyopathy (i.e., diastolic heart failure) in this population, a cardiac assessment under the direction of a cardiologist is recommended [49]. The main guide for determining an appropriate aerobic exercise intensity is self-reported symptoms (i.e., one should not exercise to a level of total exhaustion), as well as the cardiovascular response to exercise.

A secondary benefit of exercise is the impact on quality of sleep. Insomnia is a prominent issue in neurologic disorders [50]. More research on sleep is needed in the ataxia population, but in the general population it is known that people who are more active during the day have improved quality of sleep at night. The impact of physical therapy and home exercise programs on sleep among neurologic patients is a promising nonpharmacologic intervention for sleep disturbance.

Compensatory Strategies

Compensation is a common component in the rehabilitation plan of care for people with cerebellar dysfunction. In individuals living with progressive ataxias, many start using alternative movement strategies without realizing; however, others may need to be taught when and how to deploy compensatory strategies. For individuals with degenerative or acquired ataxias, where recovery of premorbid mobility is not expected, compensatory movement strategies may promote modified independence and optimal safety despite mobility impairments.

A common compensatory strategy that is often taught in rehabilitation is to slow down a movement to help maintain self-awareness of where the limb is in space. Movement complexity may be minimized by reducing the number of moving joints to achieve a desired outcome. For example, one may place an elbow on the table to stabilize a single segment of movement when drinking from a cup. Another common strategy is to encourage individuals to focus on one task at a time. Individuals with ataxia rely on cortical function to execute smooth movement. Hence, avoiding multitasking, especially while walking, may help optimize motor performance.

The use of assistive devices may promote modified independence during gait. For some individuals, the added support of a device may improve gait performance. For others, learning to coordinate the assistive device may cause further instability, and hence may not be worth pursuing. In general, people with limb ataxia and imbalance benefit more from the support of a wheeled walker rather than a cane or standard walker. A platform walker may provide proximal stability in those individuals who are more ataxic but still ambulatory. A wheelchair may be recommended for those individuals who want to remain mobile but are not safe ambulators at home or in the community.

A common goal among individuals, family members, and the health-care team is fall prevention. Interestingly, fall history has been shown to be reliably predict future falls in patients with ataxia conditions [51]. Fall status and frequency may be reliably predicted, at an accuracy of 78% and 81%, respectively, primarily based on number of falls a person has experienced in the past. Additionally, in patients who are at risk of falling, instrument-based measures of gait and mobility may provide added information on the likelihood of severe fall-related injuries [51].

Education for Lifelong Self-Management

Education of appropriate home exercises for optimizing function, as well as guidance of effective and safe mobility strategies that yield successful function, is a key role of rehabilitation therapists. Neurorehabilitation clinicians also empower individuals with ataxia with the knowledge about motor learning principles available for optimizing emerging skills, as well as knowledge about which limitations are modifiable and within the scope of rehabilitation to focus on. Individuals should seek rehabilitation intervention to optimize activities that are most meaningful to them. If individuals are making progress between rehabilitation sessions at their own pace, frequency of therapy visits may be less often. Periodic therapy sessions may be valuable for providing individuals with feedback on their progress, as well as for educating individuals on appropriate modifications to their home program.

Adherence to home exercise programs is often challenging for individuals [52]. Psychological and situational factors vary between individuals and need to be considered by clinicians when designing personalized exercise programs. Utilization of technology is an option to help optimize compliance. Activity trackers are an accessible technology that may be helpful for patients to assess their current ability and to set goals based on that ability. Smartphones are also a commonplace technology in most people's pockets now. Smartphones have access to free or low-cost apps that can monitor steps and plot daily steps over days, months, and the year. Wearable technologies can even monitor heart rates and rhythms. Smartwatches often have safety features to detect falls and provide a means for calling for help in the event of an emergency. Activity trackers and smart devices can provide individuals with cerebellar dysfunction excellent feedback on their current abilities and progress.

Another strategy to optimize compliance is to find an exercise the person enjoys, and thus would be more motivated to perform regularly. Tai Chi involves whole-body movements and weight shifting and has been found to improve balance scores in people with degenerative ataxia [53]. Yoga and Pilates are other forms of exercises that also incorporate controlled whole-body movements as well as core stability. Swimming is a safe form of whole-body exercise where one may feel safe challenging themselves without the fear of the consequence of a fall.

Virtual reality and videogame (VR/VG)-based rehabilitation is a promising intervention among the neurological patient populations, with emerging evidence in the rehabilitation literature. A coordinative training program in adolescents with early onset degenerative ataxia, using the Xbox Kinect® at home for 8 weeks, revealed significantly improved SARA scores as well as Dynamic Gait Index and Activity-Specific Balance Confidence measures [19]. The kids who exercised more frequently benefitted more and they reported having fun. Another 4-week exergaming study found significant improvements in the gait-posture SARA sub-score, but the overall outcomes were not significantly superior to conventional balance and coordination training [54]. In addition, a 12-week coordinative training program based on commercial VG systems (Nintendo Wii® and Microsoft Xbox Kinect® specifically) resulted in a 2.5-point reduction in the SARA score as well as improvements in balance control of sitting, stance, and gait in ambulatory and nonambulatory subjects with degenerative ataxia [41]. Considering the advantages of being low-cost, enjoyable, and easy to implement at home, VR/VG-based rehabilitation deserves further research to assess its effectiveness.

Controversial Interventions

The benefits of trunk and limb weighting for improving balance and gait have been debated among rehabilitation specialists over time. When discussing weighting, it is important to differentiate between trunk weighting and limb weighting, as there is different evidence for both. There is mixed evidence for trunk weighting [55], but there may be some benefit [56]. However, the use of external limb weights to control limb acceleration and inertial forces has been proven ineffective in people with degenerative cerebellar ataxias [57]. Instead, simple strategies such as slowing down movement are considered more beneficial.

There has been some limited evidence in the speech therapy literature that Lee Silverman Voice Treatment (LSVT LOUD®) exercises can improve communication in people with ataxia [58]. However, this should not be interpreted as an endorsement of large amplitude exercises for physical impairments. Ataxia is best addressed by training smaller and softer movements, rather than the large amplitude exercises trained during LSVT BIG®.

Conclusion

Evidence in the literature has shown that exercise and activity in ataxia are beneficial. Rehabilitation helps individuals gain the capacity to learn and integrate new skills through practice. Functional recovery is possible in individuals with ataxia, and the extent of recovery depends on the cause and site of the cerebellar lesion. Reinforcing movements that yield success is critical for sustained results. Functional tasks must be challenging but safe and within an individual's capabilities. Endurance training is a helpful adjunct to balance and coordination training, in addition to the known cardiovascular health benefits. Rehabilitation aims to address the functional goals that are most meaningful to individuals, as well as impairments that are most amenable to change within the scope of rehabilitation. Additionally, barriers to function, such as pain, may be addressed with the help of rehabilitation therapists and the care team. Functional assessments provide individuals with feedback and may promote compliance with home programs and safe self-management. Safe strategies, including the use of adaptive equipment, are critical for fall prevention. Individuals living with ataxia are not alone in management of their disease, with neurorehabilitation therapists as part of their care team.

Conflicts of Interest The authors have no conflicts of interest.

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Epidemiology of Cerebellar Disorders

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Abstract In this updated chapter, we briefly describe 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 health-care systems in various parts of the world, inaccurate classification of disorders in published studies, broad inclusion criteria, or simply the rarity of the disorder. Information about the prevalence, incidence, or number of cases is important for the planning and provision of services to address the needs of affected individuals and their families. 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

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Introduction

In the first edition of this chapter, we described the epidemiology of cerebellar disorders and where possible provided information on their global and local prevalence and incidence and risk factors [192]. For this second edition, we located recent research and updated the relevant sections of this chapter based on this literature. *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 and their families. This information 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, 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 aide 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, using the following search terms: [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 for cerebellum OR cerebellar). The search was restricted to records published in English and ending December 2016. For the first edition chapter [192], we located 366 references, and our initial examination of the records revealed that 29 references to book, chapters, and sections, and 337 references to articles were relevant to epidemiology or risk factors. For this updated chapter, we replicated our search, limited our search to publications beginning in January 2017 to February 2022, and reviewed an additional 515 titles and abstracts, using Covidence Systematic Review Software [227], and the full text of 83 sources were reviewed.

We selected studies providing estimates of prevalence and/or incidence for a specified population in a defined geographical region, and/or associated risk factors. 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) the article must be 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 nonconsensus, third (and sometimes fourth) reviews were 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. 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. Quality assessments of the studies were not conducted.

Results

Many cerebellar disorders are described as rare, very rare, or extremely rare. According to the consortium of European partners [38], "rare" is defined as affecting 1 per 2,000 people. Similarly, the United States Rare Diseases Act of 2002 [37] 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.

Ataxia

The word ataxia is derived from the Greek word "a taxis," which means "without order." Individuals 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 [124]. Ataxia can negatively influence a person's ability to walk, sit, and stand [193]. The prevalence of ataxia in children is 26 per 100,000 [124] and lifetime prevalence rate is 50 per 100,000 (see [52]), but these prevalence estimates vary depending on the type of ataxia or region studied. The most common types of ataxias are cerebellar (including hereditary and nonhereditary ataxias), sensory, and vestibular. Ataxia is associated with numerous conditions, including the presence of cerebellar tumors, Joubert syndrome and related disorders (JSRD), Gómez-López-Hernández (GLH), rhombencephalosynapsis, cerebellitis, and cerebellar stroke (see below).

Hereditary Cerebellar 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 based on 22 studies, reporting on 14,539 patients from 16 countries, published between 1983 and 2013, Ruano et al. [173] 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 [153] and 5.3 per 100,000 [10]. 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 et al. [49] found that the prevalence of AR-HCA was 2.3 per 100,000. On average, individuals were 32 years (*Range*: 4–71 years) and were diagnosed at the age of 9 years (*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 [57]). See Table 1 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 [57]. Friedreich ataxia is the most common form of AR-HCA in the world (see [112, 173]). 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 [57]. 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 HCA identified among 135 patients with cerebellar ataxia from March 1993 to March 1999 in Dr. Shariati Hospital, University of Tehran [125]. 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) [49]. Consanguineous marriage is an important risk factor for autosomal recessive ataxias [51, 82, 146, 160, 229].

Autosomal Dominant Ataxia

In their review, Ruana and colleagues [173] 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

Table 1 Prevalence, incidence, and/or number of cases reported in studies examining the epidemiology of ataxia

	Study details				Population					
First author (date)	Research design	Country (region)	Study period	Data source	Age (years)	Sex	*N*	Prevalence	Incidence	*N* or % of cases
Anheim [10]	Retrospective	France (Alsace)	2002–2008	Hospitals	–	M & F	95	AR = 5.3/100,000	–	–
Erichsen [49]	Cross-sectional	Norway (southeast)	2002–2008	Population based	0–80	M & F	171	AD = 4.2/100,000; AR = 2.3/100,000	–	–
Farghaly [52]	Community based	Egypt	–	Door-to-door survey	4–72	M & F	62,583	Acquired ataxia = 27.16/100,000	–	17 (7 F; 10 M)
Ono [138]	Community-based	Japan	–	Mailed out survey	0–20	–	–	Childhood-onset ataxia = 0.93/100,000	–	–
Polo [153]	Retrospective	Spain (northern)	1974–1986	Hospitals, families		M & F	54	AR = 7.2/100.000	–	–
Nafissi [125]	Retrospective cross-sectional	Iran	1993–1999	Dr. Shariati Hospital, University of Tehran	6–73	M & F	135	–	–	HCA: 15
Ruano [173]	Review (22 studies)	16 countries	1983–2013	Multisource, hospitals, families, registries	–	M & F	14,529	AD = 2.7/100,000; AR = 3.3/100,000	–	–
Salman [179]	Retrospective	Canada (Manitoba)	1991–2008	Children's hospital	0–16	M = F	184	Chronic ataxia = 2.4/10,000	Chronic ataxia = 3.2/100,000	9 cases of mitochondrial disease

(continued)

Table 1 (continued)

	Study details				Population					
First author (date)	Research design	Country (region)	Study period	Data source	Age (years)	Sex	*N*	Prevalence	Incidence	*N* or % of cases
Velázquez-Pérez [225]	Retrospective	Cuba	2017–2018	Centre for the Research and Rehabilitation of Hereditary Ataxias (CIRAH)	–	–	1001	Hereditary ataxia = 8.91/100,000	–	–
Vural [229]	Cohort study	Turkey	2 decades	Neurodegeneration Research Laboratory (NDAL)	0–75	–	1296 individuals and their families	–	–	AD = 43.7%; AD = 39.0%; sporadic cases = 36.6%

Note*: AD* Autosomal dominant, *AR* autosomal recessive, *HCA* hereditary chronic ataxia, *F* female, *M* male, *yrs* years

100,000). No cases of AD-HCA were found among 16 Italian patients with hereditary ataxia [55], whereas other work conducted in Portugal suggests a prevalence rate of 5.6 per 100,000 population [40]. Prevalence rates in multisource population-based surveys (e.g., [40]) or in the registry studies (e.g., [215]) were higher than genetic center-based studies ranging from 1.6 to 2.5 per 100,000. For example, Velázquez-Pérez et al. [225] found the prevalence of hereditary ataxias in Cuba to be 8.91 per 100,000 using data obtained from a registry. In the Netherlands, the prevalence of the AD-HCA is at least 3 per 100,000 [221]. In Japan, Ono et al. [138] estimated the prevalence of childhood-onset ataxia to be 0.93 per 100,000 children. 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 [49]. The mean age of the sample of cases of AD-HCA was 57 years (*Range*: 13–94 years) without any gender difference after adjustment for age [49].

Among individuals with AD-HCA, spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease, may be the most common [127, 206, 224, 229], followed by SCA2, SCA6 (see [173]), SCA7, and SCA10 [206], but prevalence estimates depend highly on the country or region within a country studied. For example, SCA3 is most common in China, Thailand, and Japan, whereas SCA2 is most common in India [21, 224] and Cuba [167, 225]. Interestingly, SCA1 and SCA3 are the most common subtypes in the Buriram province in northeast Thailand [231]. Nearly 50 subtypes of SCA have been identified (each presenting with classic progressive ataxia along with a differentiating non-ataxia symptom) [224] and the genetic mutations associated with each subtype are continually being identified [143] (see Table 1).

X-Linked Ataxia and Ataxia Due to Mitochondrial Mutations

Little information is available on the epidemiology of X-linked and mitochondrial HCA, which may be because the required genetic testing for the diagnosis of these conditions is not performed. Few cases of X-linked ataxias or ataxias linked to mitochondrial mutations have been described in the research literature [56]. For example, in a retrospective study using multiple sources of data of children examined at Children's Hospital in Manitoba, Canada, Salman et al. [179] reported nine cases of intermittent or chronic ataxia 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 1).

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 [86, 98]. 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 et al. [52] 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 a male-to-female ratio of 2.1:1 (see Table 1).

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 et al. [208] reported that postinfectious 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. Evidence from other research, however, suggests that varicella and other infections are strongly associated with ACA [61, 97, 222]. In a case–control study to examine risk factors for ACA in children in Children's Hospital of Nanjing Medical University, China, Zhu et al. [240] found that age, infection, vaccination, head trauma, and surgeries for intussusception, indirect inguinal hernia, and congenital gastrointestinal malformation were independent risk factors for ACA. Postinfectious cerebellar ataxia accounts for up to 40% of ACA in preschool children (aged 1–4 years), which is followed by toxic ingestion (i.e., 30% of ACA cases) [193]. Strokes (ischemic or hemorrhagic) and medications are other potential cause of ACA, particularly in elderly individuals [52]. 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 [57].

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 [8]. The comorbidity of ASD and intellectual disability (ID) is relatively low, with approximately 31% of US children with ASD being identified as having ID (i.e., IQ $\leq$ 70 [14]). The cerebellum is one of the key brain regions affected in autism [19].

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 1 person in 160 [234]. Many epidemiological studies from developed countries have investigated ASD prevalence, but 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. Although

earlier European studies reported ASD prevalence estimates of 1 in 2500 children across all ages in the population [64], more recent estimates of ASD prevalence based on large survey data suggest that 1–2% of all children are affected [104, 187]. For example, a UK school-based survey reported 99 per 10,000 children [16]. 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) [238]. 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 [238]. Other studies from Europe, North America, and Asia also reported prevalence estimates of higher than 2% [14, 96, 168].

The Autism and Developmental Disabilities Monitoring (ADDM) Network is an active surveillance system in the United States (US), 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) [14] and 23.0 per 1,000 (1 in 44) in 2018 [114]. In both these reports, there was variation in the reported prevalence estimates by sex and racial/ethnic background. ASD was 3–5 times more prevalent in boys than in girls, depending on the geographic region. White children were also 30% more likely than non-Hispanic black children to be identified with ASD in the 2014 study but prevalence was similar across racial and ethnic groups in the 2021 study. An exception was that the ASD prevalence was higher for Indigenous children than White children (29.0 vs 21.2 per 1,000) [114]. The median age at first ASD diagnosis was 53 months in the 2014 study, which decreased to 50 months in the 2021 study.

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 (i.e., 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 prevalence among children aged 2–14 years ranged from 9.7% to 14.6% [141]. A more recent estimate of the prevalence of ASD in Canada in 2015 was reported by the National Autism Spectrum Disorder Surveillance System [158] to be 1 in 66 children and youth (or 15.2 per 1000) aged 5–17 years. Age differences in prevalence were consistent with other regions in that more boys (1 in 42) than girls (1 in 165) were diagnosed with ASD. See Table 2 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, and better reporting of cases and surveillance systems. The

Table 2 Prevalence of autism spectrum disorders

	Study details				Population			
First author (date)	Research design	Country (region)	Study period	Data source	Age (years)	Sex	*N*	Prevalence
ADDM Network Surveillance Year 2010 Principal Investigators [14]	–	The United States	2010	ADDM Network	8	M & F	–	14.7/1000 (overall)
Baron-Cohen [16]	Cross-sectional	The United Kingdom	–	Special Educational Needs (SEN) register & Survey	5–9	M & F	11,700	9.9/1000
Maenner [114]		The United States	2018	ADDM Network				23.0/1000 (overall)
Ouellette-Kuntz [141]	Retrospective	Three Canadian provinces	2008–2010	National Epidemiologic Database for the Study of Autism in Canada (NEDSAC)	2–14	M & F	–	10.6/1000
Public Health Agency of Canada [158]	–	All Canadian provinces and territories	2015	Data from education, social services, and health sectors	5–17	M & F		15.2/1000 (overall)
World Health Organization [234]	–	Global	–	–	–	–	–	6.25/1000
Zablotsky [238]	Cross-sectional	The United States	2011–2014	Population based	3–17	M & F	43,283	22.4/1000

Note: *ADDM* Autism and Developmental Disabilities Monitoring, *F* female, *M* male, *yrs*years

observed increase in ASD prevalence could also be as a 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 influence early brain development, leading to ASD [41, 130]. For example, a higher concordance between monozygotic compared to dizygotic twins is consistently shown, suggesting a genetic link for ASD [109]. Other researchers have estimated that approximately 10–15% of persons with autism have a specific genetic mutation (see [3]).

Recent epidemiological studies revealed a positive association between increasing parental age at conception and ASD risk in offspring (see [100] for a review). In contrast, a review of US data led to the conclusion that parental age is a very small contributor to the observed increases in the prevalence of ASD [163]. 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 [89, 100]. Mothers exposed to high levels of pesticides and air pollution may also be at higher risk of having children with ASD (e.g., [81]), although the evidence for this assertion has been described as limited and of moderate strength (see [105]). Interestingly, maternal smoking is also not associated with increased ASD [170]. 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 [47]. 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 [113] for a review). The information available on risk factors associated with ASD clearly suggests that there is no single cause of autism.

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 (CNS) tumors each year (see [22]). In the United States, over 3000 children under the age of 20 years are diagnosed with a brain or spinal cord tumor annually [203]. The incidence of brain tumors in children is estimated at 2.76–4.28 per 100,000 children per year [202]. In Thailand, the incidence of CNS tumors in children ages 0–15 years of age has been reported to be 7.5 per million for the period of 2003–2005 but much higher at 13.24 per million in 2011–2012 [154]. 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 [24, 202]. In the following subsection, we review the existing epidemiological information on medulloblastoma, one of the most common malignant CNS tumors in children.

Medulloblastoma

Medulloblastomas, which typically arise in the cerebellum, are the most common malignant CNS tumor in children and the second most common pediatric brain neoplasm. Medulloblastoma accounts for 12–25% of all CNS tumors in children [203, 233] and present at approximately 5–7 years of age, and occur more frequently in boys than in girls [13, 111, 116, 137, 154, 194]. Of newly diagnosed cases of medulloblastoma, 25% occur in individuals aged 19 years and older [166]. The earlier incidence estimates of medulloblastoma brain tumor was 9.6 per million in children and 0.54 per million adults [65, 210]. The European annual incidence rate for primitive neuroectodermal tumors (PNET; morphologically similar tumors arising in other areas of CNS) was reported to be 6.5 per million children (age 0–14 years) for the period 1988–1997 [149]. The incidence rates of medulloblastoma and PNET are stable from birth to 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 et al. [202] 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%), brainstem glioma (7.2%) and miscellaneous pathologies (e.g., dermoid and tuberculoma) (7.2%).

Ahmed et al. [5] 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 identified, 71.6% were males and 28.4% were females (i.e., male-to-female ratio was 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 aged 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, 66.7% were infratentorial tumors, and 70.4% of the infratentorial tumors were medulloblastomas. Consistent with other research [194], Ahmed et al. [5] 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 et al. [13] found that medulloblastomas were the second most common type of brain cancers (11.4% of cases) among 1403 tumors that were identified in children (aged 0–18 years) diagnosed between 1990

Table 3 Prevalence, incidence, and/or number of cases reported in studies examining the epidemiology of cerebellar tumors

	Study details				Population					
First author (date)	Research design	Country (region)	Study period	Data source	Age (years)	Sex	*N*	Prevalence	Incidence	*N* or % of cases
Ahmed [5]	Cross-sectional	Pakistan	1989–1998	Hospital database	*M* = 8.8; *range*: 5–9	M = 71.6%; F = 28.4% M/F = 2.5/1	81	–	–	–
Asirvatham [13]	Retrospective cohort	India	1990–2004	Pathology and medical records	*M* = 10.9; *range*: 0–18	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 [33]	Retrospective cohort	Singapore	1997–2005	Singapore Children's cancer registry	0–15	M & F	39	–	–	Medulloblastoma/ PNET = 40.7% of tumors
Giordana [65]	Retrospective cohort study	Italy	1976–1995	Hospital charts and operating-room books from 5 neurosurgical units	16–69	M & F	4.3 million	–	All ages: 0.5/ million/yr.; M: 0.82/million/ yr.; F: 0.28/ million/yr.; Highest incidence for 16–19-yr olds (2.33/million/ year)	45 (32 M; 13 F) cases of medulloblastoma

(continued)

Table 3 (continued)

	Study details				Population					
First author (date)	Research design	Country (region)	Study period	Data source	Age (years)	Sex	*N*	Prevalence	Incidence	*N* or % of cases
Johnston [88]	–	Canada	1990–2009	Canadian Pediatric Brain tumor consortium	0–14	M & F	–	–	All ages: 4.82/million; <5 yrs.: 7.92/million; 5–14 yrs.: 3.64/million	574 cases
Lombardi [111]	Case–control study	The United States	1998–2011	California cancer registry	0–5	52 F; 105 M	667 cases of childhood CNS tumors; 123,158 controls	–	–	157 cases of medulloblastoma
Peris-Bonet [149]		Europe	1978–1997	ACCIS database	0–14	M & F	19,531		ASR for CNS tumours (1988–1997) = 29.9/million; ASR for PNET = 6.5/million (1988–1997)	–
Pongtanakul [154]	Retrospective cohort study	Thailand	2003–2005; 2011–2012	National childhood CNS tumor registry	0–15	M/F = 1.5/1	–	–	All CNS tumors 2003–2005: 7.5/million; 2011–2012: 13.24/million	Cases of medulloblastoma/PNET: 2003–2005 = 90/300; 2011–2012 = 36/168
Roldán [169]	Retrospective cohort study	Canada	1975–1996	Alberta cancer registry	Age of diagnosis: $M_{children} = 7.0$; $M_{adults} = 29.2$	M > F	2.8 million	–	–	49 cases of medulloblastoma/PNET

Smoll and Drummond [194]	Retrospective cohort study	The United States	1973–2007	Surveillance, epidemiology, and end results (SEER) database	Children (1–9 yrs) 10 times more affected by medulloblastoma and 4.6 times more by PNET	M/F = 1.58/1	–	–	Medulloblastoma = 1.5/million population, PNET = 0.62/million population	1372 cases of medulloblastoma, 530 cases of PNET
Tabatabaei [202]	Retrospective cohort study	Iran	1981–2011	Patient records	1–14	M > F	84	–	Brain tumors = 2.76–4.28/100,000 children per year	Medulloblastoma in 42.8% of cases
Thorne [210]	Retrospective population-based study	England (Southwest & Northern)	1976–1991	Bristol registry of childhood Cancer	0–14	M & 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
Williams [233]	Retrospective cohort study	Australia	2015–2019	Queensland Children's hospital	0–19	–	221 cases of CNS tumors	–	–	29 cases (or 13.12%) of medulloblastoma

Notes*: ASR* age-standardized incidence rate, *CNS* central nervous system, *F* female, *M* male, *yrs* years, *PNET* primitive neuroectodermal tumor

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 et al. [33] 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 were the most common type of brain tumor in the sample, accounting for 40.7% of all brain tumors. In Australia, Williams et al. [233] examined the 221 cases of newly diagnosed CNS tumors from January 2015 to December 31, 2019 and found that 13.12% were medulloblastoma.

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 [194] 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, and children (1–9 years of age) were 10 times more likely to be diagnosed with these tumors than adults (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 an medulloblastoma, and this was significant in children and adults [194].

Roldan et al. [169] 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. Of the 49 cases of medulloblastoma or PNET identified, the majority (61%) were children and male. The mean age at the diagnosis for children was 7 years and for adults it was 29.2 years. A 2014 Canadian study reported an overall incidence of medulloblastoma to be 4.82 per 1,000,000 children aged 14 years and younger using data collected from 1990 to 2009 by way of a questionnaire completed by 16 member centers of the Canadian Pediatric Brain Tumor Consortium [88]. The authors also showed a higher incidence of medulloblastoma in male children (1.7:1) and an increase in incidence over the first three periods (1990–1994: 24%, 1995–1999: 27.5%, 2000–2004: 27.7%), followed by a decrease in incidence during 2005–2009 (21%).

Although some genetic disorders (i.e., Gorlin syndrome, Turcot syndrome, Li-Fraumeni syndrome [LFS]) are associated with an increased risk of medulloblastoma, for most patients the etiology is unknown [228]. Because the highest incidence rate is reported during childhood, very early life experiences may be contributing factors in the development of brain tumors [116]. A meta-analysis conducted by Harder et al. [72] 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 11-fold increased risk of malignant nervous system tumor compared to children whose mothers did not have such a history during their pregnancy [53]. Lack of social contact in the first year of life is associated with increased risk of developing a CNS tumor in childhood, and the effect is greater for medulloblastoma/PNET [73].

The role of diet as a potential risk or protective factor in brain tumors has been investigated (e.g., [15, 31, 152]). 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., [162]). 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 products, and oil products; however, yellow-orange vegetables, fresh fish, and grains reduced the risk significantly [152].

Studies based on a very small sample size have also reported that exposure to electromagnetic fields are a potential risk factor for childhood brain tumor [95]. 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 [218]. A 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 [108]. 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 [108].

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 [191]. The researchers reported very weak associations between PNET for any of the pesticide classes or exposure sources considered. However, Rosso et al. [172] found an association between household exposure to chemicals and medulloblastoma/PNET in children registered with Children's Cancer Group (the United States and Canada), particularly for pesticides used in lawn care. A US study using data obtained from the California Cancer Registry, Lombardi et al. [111] found an association between medulloblastoma in children 0–5 years of age and exposure to four pesticides: chlorothalonil, propiconazole, dimethoate, and linuron. 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 [39].

Fetal Alcohol Spectrum Disorders

Fetal alcohol spectrum disorders (FASD) are a group of conditions that occur 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) [165]. Alcohol has irreversible effects on CNS, 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 [165]. Growth deficiency (height and weight), CNS 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 individuals with FASD [20]. However, brain malformations are variable in FASD, as evidenced by an examination of 174 cases identified with prenatal exposure in a retrospective survey of autopsies at the Health Sciences Centre in Winnipeg, Canada [85] and blinded review of the MRIs of 164 individuals with prenatal exposure (and 163 controls) in Edmonton, Canada [214].

A systematic review of FASD prevalence found significant variations in the reported prevalence estimates across the reviewed studies [139]. Ospina and Dennett [139] classified 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 was 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%. Most studies that have examined estimates of FASD prevalence in Indigenous populations were conducted in Canada. The pooled estimate of FAS prevalence in Aboriginal people based on six studies was 0.2%, or two FAS cases per 1,000 population. The FASD prevalence in other specialized settings, such as 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 et al. [155] 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) [107, 226]. 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 [237].

Our search of the literature provided estimates of prevalence and/or incidence, or number of cases resulted in one article describing one new case of complete primary cerebellar agenesis [237]. Yu et al. [237] described the clinical presentation and subsequent imaging tests of a 24-year-old female, who was married and having a daughter, 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 [188, 190, 207, 211, 223, 226, 236]. Interestingly, some individuals with total or subtotal cerebellar agenesis are asymptomatic and have typical neurobehavioral, mental, and physical functioning [190]. See Table 4 for a summary of statistics.

Dandy-Walker Malformation

Dandy-Walker malformation (DWM) is a complex developmental anomaly involving fourth ventricle and cerebellum, characterized by an enlargement of the fourth ventricle, vermian agenesis (partial or complete), and posterior fossa cysts [83, 129, 164]. Hydrocephalus is a common finding in DWM cases, and can lead to death if not treated quickly [120]. Epidemiological studies of DWM have been conducted in the United States [101, 119], Italy [45], Saudi Arabia [71], and across Europe more broadly [180]. All studies had retrospective designs, with sample sizes ranging between 129 and 14,599. Di Bella and Pizzo [45] examined the health records of 5000 children referred to a pediatric radiology unit at the University Hospital of Catania, Italy for diagnostic procedures over a 10-year period (1999–2009). The authors found 16 cases of DWM, ranging in age from 1 month to 9 years (10 males, 6 females) and estimated the prevalence of DWM 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 [71] reported that 22 infants were identified with DMW (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 [136] identified all infants diagnosed with DWM during an 11-year period (1989–1999) from a cohort of 45,274 live births.

Table 4 Prevalence, incidence, and/or number of cases reported in studies examining the epidemiology of cerebellar malformations

	Study Details				Population					
Disorder First author (date)	Research design	Country (region)	Study period	Data source	Age (years)	Sex	*N*	Prevalence	Incidence	*N* or % of cases or families
Cerebellar agenesis										
Sener [190]	Case report	Texas, USA	–	University of Texas Health Science Center	58	F	–	–	–	1
Sener [188]	Case report	–	–	–	6	1 F; 1 M	–	–	–	2
Tekin [207]	Case report				7	F	–	–	–	1
Timmann [211]	Case report	Germany	–	–	59	F	–			1
Van Hoof and Wilmink, [223]	Case report				46	M	–	–	–	1
Velioglu [226]	Case report	–	–	–	22	M	–	–	–	1
Yu [237]	Review, case report	China	–	General Hospital of Jinan Military Command	24	F	–	–	–	1
Dandy-Walker malformation (DWM)										
Di Bella and Pizzo [45]	Retrospective	Italy (Catania)	1999–2009	–	Pediatric	–	5000	32/10,000	–	16
Hakami and Majeed-Saidan [71]	Retrospective analysis of prospectively collected data	Saudi Arabia	2001–2010	Neonatal Intensive Care Unit, Riyadh Military Hospital	–	–	94,210	2.3/10,000	–	–
Kontopoulos [101]	Retrospective	Florida, USA	1997–2005	–	–		600 monochorionic twins	–	1/8000–100,000 live births	10

McClelland [120]		The United States (22–36 states)	1997–2003	Kids' Inpatient Database	–		–		1.36/1000	–
Ohaegbulam and Afifi [136]	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; M: (1.24/10,000), F: (0.78/10,000)	–
Santoro [180]	Retrospective	17 countries	2002–2015	28 population-based registries	Pediatric	–	8,028,454 surveyed births	6.79/100,000	–	562 DWM, 172 DW variant
Joubert syndrome and related disorders (JSRD)										
Akhondian [6]	Case report	Iran	–	–	12, 10, 3	2 F; 1 M	3	–	–	3 in one family
Brancati [27]	Review	–	–	–	–	–	–	–	1:18,000–1:100,000	
Hakami and Majeed-Saidan [71]	Retrospective analysis of prospectively collected data	Saudi Arabia	2001–2010	Neonatal Intensive Care Unit in Riyadh Military Hospital	–	–	94,210	1.7/10,000	–	–
Nuovo [134]	Cross-sectional	Italy	2018	46 Italian centers that diagnosis, care for, and research of JSRD	0–19; 20+	128 F; 146 M	284	Total: 0.47/100,000; F: 0.41/100,000; M: 0.53/100,000; 0–19 yrs.: 1.7/100,000	–	–

(continued)

Table 4 (continued)

	Study Details				Population					
Disorder First author (date)	Research design	Country (region)	Study period	Data source	Age (years)	Sex	*N*	Prevalence	Incidence	*N* or % of cases or families
Lissencephaly and cerebellar hypoplasia										
Koul [102]	Case report	Oman	1993–2003	NR	15 days to 6 yrs	4 F; 8 M	40	–	–	12; family history of developmental delay in 2/7 cases
Howley [79]	Case–control study	The United States	1997–2011	National Birth Defects Prevention Study (NBDPS)				1.30/100,000 births		87
Ozyurek and Kose [144]	Case report	Turkey	–	–	3	M	1	–	–	1
Pontocerebellar hypoplasia										
Alkan [7]	Retrospective study of MRI and CT images	Turkey	2002–2008	–	8.9 (30 wks gestation – 17 yrs)	22 F; 23 F	45	–	–	12
Grellner [69]	Case report	Germany	–	–	1.5	M	1	–	–	1
Zafeiriou [239]	Retrospective case study	Greece	–	Tertiary hospital	24–28 wks (*M* = 25.8 wks)	7 F; 5 M	12	–	–	12 observed in extreme prematurity
Gómez-López-Hernández syndrome (GLH)										
Abdel-Salam [2]	Case report	Egypt	–	–	24 mths	M	1	–	–	1

Choudhary [34]	Case report	India	–	–	4	M	1	–	–	1
De Mattos [43]	Case report	Brazil	–	–	Minutes old	M	1	–	–	1
Ebrahim Ali and Hajeri [48]	Case report	Bahrain	–	–	6	M	1	–	–	1
Erzin [50]	Case report	Turkey	–	–	24	M	1	–	–	1
Fernandez-Jaen et al. [54]	Case report	Spain	–	–	14 mths; 6	M	2	–	–	2
Gomy [66]	Case report	Brazil	–	–	12, 29	M	2	–	–	2
Kobayashi [99]	Case report	Japan	–	–	42 wks gestational age; then at 4 and 39	F	1	–	–	1
Perrone [150]	Case report	Brazil	–	–	27 wks gestational age, then at 6	F	1	–	–	1
Poretti [156]	Case report	Switzerland	–	–	38 weeks gestation to 39 yrs	2 F; 2 M	4	–	–	4
Rush [176]	Case report	The United States	–	–	4–11	3 F; 1 M	4	–	–	4
Saricam [181]	Case report	Turkey	–	–	16	F	1	–	–	1
Schell-Apacik [186]	Case report	Germany	–	–	3.67, 15.67	M	1	–	–	1
Sukhudyan [201]	Case report	Armenia	–	–	1.5–20	M	6	–	–	6

(continued)

Table 4 (continued)

	Study Details				Population					
Disorder First author (date)	Research design	Country (region)	Study period	Data source	Age (years)	Sex	*N*	Prevalence	Incidence	*N* or % of cases or families
Rhombencephalosynapsis (RS)										
Arisoy [12]	Case report	Turkey	–	–	19 wks		–	–	–	1 fetus
Passi and Bhatnagar [148]	Case report	India	–	–	4	M	1	–	–	1
Weaver [230]	Retrospective case report	The United States	–	–	10 mths – 10	3 F; 6 M	9	–	–	6 of RS; 3 of partial RS
Sener [189]	Retrospective review of MRI examinations	Turkey	–	–	1 day – 18	–	3000	13/10,000	–	6 (3 mths–8 yrs., 3 F, 3 M)
Utsunomiya [219]	Case report	Japan	–	–	3, 4	2 M	–	–	–	2
Chiari malformation										
Bogdanov [23]	Retrospective study	Russia	1998–2008	Archival data of hospitalizations			29,008 hospitalizations	30/100,000	–	–
Ciaramitaro [36]	Retrospective cohort study	Italy	2011	Interregional Piemonte and Valle d'Aosta Rare Disease Registry	All ages	292 F; 144 M	–	7.74/100,000 F: 10.17/100,000 M: 5.13/100,000	3.08/100,000 F: 3.75/100,000 M: 2.36/100,000	–
Di Bella and Pizzo [45]	Retrospective	Italy (Catania)	1999–2009	–	Children	M/F	5000	32/10,000	–	12 (0.24%)

Dughal [46]	Prospective observational	Pakistan (Lahore)	2010–2012	–	Infants with anomalies	33 F; 47 M	80	–	–	2 (3%)
Ghavami and Abedinzadeh [62]	Prospective observational	Iran (East Azerbaijan)	2005–2008	–	–	–	22,500 pregnant women	–	–	41 fetuses
Horn [78]	Retrospective review	The United States	2003–2012	Nationwide Inpatient Sample (NIS)	All ages		520,561 cases	–	–	CMI = 305,726; CMII = 119,632; CMIII = 15,540; CMIV = 79,663
Lee [106]	Retrospective review of surgical records	Korea	1991–2012		12–250 mths	–	–	–	–	54
Meadows [121]	Retrospective analysis of MR images	The United States (Maryland)	1994–1997	Imaging report database, Johns Hopkins Hospital	0–70	–	22,591	7.8/10,000	–	0.77% CMI
O'Reilly and Torreggiani [135]	Retrospective cohort study	Ireland	Over 24 months	–	15–83	82 F; 65 M	147	–	–	Asymptomatic CMI: 2%
Sakushima [177]	Survey	Japan	2008–2009	Nationwide postal survey	38 ± 23.5	56.5% F; 42.1%M	708	–	–	CMI: 48.0%; CMII: 8.1%
Schanker [185]	Case report	The United States	–	–	20–62	5 F; 1 M	–	–	–	CMI: 3 families pairs (2 mother–daughter; 2 father–daughter)

(continued)

Table 4 (continued)

	Study Details				Population					
Disorder First author (date)	Research design	Country (region)	Study period	Data source	Age (years)	Sex	*N*	Prevalence	Incidence	*N* or % of cases or families
Agrawal [4]	Case report	India	–	Datta Meghe Institute of Medical Sciences	3 months	M	1	–	–	1
Anik [11]	Case report	Turkey	–	Kocaeli University	5 months	F	1	–	–	1
Chowdhary [35]	Case report	Saudi Arabia	–	King Faisal University	1 wk. to 5 mths	3 F; 1 M	4	–	–	4
Friede [58]	Case report	Switzerland	–	University of Zürich	2 mths, 8 yrs., newborn	1 F; 2 M	3	–	–	3
Goulart [67]	Case report	Brazil	–	–	Born at term	1 F	1	–	–	1
Krishnamurthy [103]	Case report	India	–	Maulana Azad Medical College	7 months	M	1	–	–	1 case
Nicolas-Jilwan [131]	Case report	Saudi Arabia	–	–	Born at term; 5 days	1 M	1	–	–	1 case
Poretti [157]	Case report	The United States	–	The Johns Hopkins School of Medicine	4 yrs	F	1	–	–	1 case

Notes: *CMI* Chiari Type I malformations, *CMII* Chiari Type II malformations, *DWM* Dandi-Walker malformation, *F* female, *M* male

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).

Using data obtained from the European population-based registries of congenital anomalies belonging to the European Surveillance of Congenital Anomalies (EUROCAT) network, Santoro et al. [180] found 734 cases of DWM and DW variant among 8,028,454 total births. The overall prevalence of DWM and DW variant was estimated at 8.85 per 100,000 and 6.79 per 100,000 births for DWM alone. The authors found significant differences in prevalence between regions and countries with Wales (14.12 per 100,000) and Ukraine (11.40 per 100,000) having some of the highest rates in the study.

In the United States, the incidence of DWM was estimated at 1.36 per 1,000 in a study examining data from the Kids' Inpatient Database containing information from hospitals in 22–36 states covering the years 1997–2003 [120]. Another US study reported that the incidence of DWM in complicated monochorionic twins was approximately 200 times higher than that expected for the general population [101]. DWM 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 birth weight, and twin births [161], but these findings have been inconsistent [178] (see Table 4).

Joubert Syndrome and Related Disorders

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 ID [90, 91, 128]. Nephronophthisis (NPHP) or cystic renal dysplasia and liver involvement have been observed in approximately one-quarter [213] and nearly one-half [200] of cases of JSRD, respectively. An important malformation is the *molar tooth sign* (MTS) – a pathognomonic midbrain–hindbrain malformation [27, 115].

Globally, the estimated incidence of JSRD ranges from 1 per 80,000 to 1 per 100,000 live births, although some researchers suggest that this range may underestimate the actual number of cases of the syndrome [27]. For example, Srour [196] and Srour et al. [197] suggested that there is a higher prevalence of Joubert syndrome within the French–Canadian population, particularly in the Saint-Lawrence region of the province of Quebec, Canada. Akhondian et al. [6] identified and described the same presentations of JSRD in three family members in Iran. In a Saudi Arabian study, Hakami and Majeed-Saidan [71] (see also DWM) found 22 cases of JSRD (incidence: 1.7 per 10,000 live births). Nuovo et al. [134] examined the clinical-genetic database containing data from 46 Italian centers active in the diagnosis, care, and research of JSRD, and estimated the crude prevalence for total, females, and males were 0.47, 0.41, and 0.53 per 100,000 population, respectively. Prevalence

increased to 1.7, 1.62, and 1.80 for total, females, and males when the authors focused on individuals aged 0–19 years. Both parents of 80% of the cases were from Italian origin. Thus, it appears that ethnicity can be a risk factor for the condition.

Lissencephaly and Cerebellar Hypoplasia

Lissencephaly and cerebellar hypoplasia (LCH) is a rare autosomal recessive disorder in which cerebellum, hippocampus, and brainstem are affected [77]. Generally, lissencephaly is caused by impairment in neuron migration, which is essential for development of cerebellar cortex. Consequently, the cerebellar cortex becomes smooth (i.e., lacks folia and sulci) [198]. 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 ID and delayed development. Prevalence of LCH is largely unknown [151]. Koul et al. [102] 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 [144] but the type of lissencephaly was not reported. Howley et al. [79] examined National Birth Defects Prevention Study (NBDPS) data collected in ten US states from 1997 to 2011. The authors found 87 eligible cases with non-syndromic cerebellar hypoplasia, resulting in an overall birth prevalence of 1.30 per 100,000 births. Howley and colleagues also found that cases were more likely to be from multiple pregnancy births, to be born preterm, and to have low birth weight. In addition, the number of cases of cerebellar hypoplasia increased in later years of the NDBPS. Zika virus infection in pregnant mothers has also been suggested as a risk factor for lissencephaly [42].

Pontocerebellar Hypoplasia

Pontocerebellar hypoplasia (PCH) is a group of prenatal onset, autosomal recessive, neurodegenerative disorders that affects brain development [126]. Characteristic features of PCH include atrophy of brainstem, particularly pons (pontine nuclei), cerebellum (with a dragonfly pattern; [175]), movement problems, ID, and communication difficulties (i.e., lacking ability to speak) [174]. Affected individuals die during infancy or childhood [17] before the age of 6 years [199].

The condition appears to affect males and females similarly and has been observed in infants born extremely prematurely [239]. About 100 cases of PCH have been reported in the literature [133]. In their retrospective study of the magnetic resonance imaging (MRI) and computed tomography images from 45 children (22 girls, 23 boys; 30 weeks–17 years of age) of cerebellar malformation, Alkan et al. [7] identified 12 cases with cerebellar hypoplasia. Grellner et al. [69] described one case of PCH Type 2 – a 1.5-year-old boy who had severe psychomotor delay, and dyskinesia and epileptic seizures. Given that the genetics of PCH are largely understood, genetic carrier screening in a specific community in the Netherlands

took place to identify high-risk couples for having children with PCH [117]. Between September 2012 and 2013, Mathijssen et al. [117] identified that 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

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 [99]. 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 [54]. Although ID is typically observed, individuals with normal cognitive function have been described in the literature (e.g., [123]).

According to Perrone et al. [150] review, 57 cases of GLH syndrome have been identified in the literature. Cases have been described in Armenia [201], Bahrain [48], Brazil [43, 150], Egypt [2], Germany [186], India [34], Japan [99], Spain [54], Switzerland [156], and Turkey [50, 181]. Because so many cases described have been found in Brazil, a "founder effect" has been suggested for GLH [43]. Several researchers have argued that GLH may not be as rare as has been previously suspected and suggest that it is underrecognized in the pediatric population because clinical presentation varies in severity [99, 123, 156].

Suggested risk factors for GLH include smoking and cannabis use, and the use of other drugs (i.e., valproate, ethosuximide, misoprostol) by mothers during pregnancy [150, 204]. No specific mutation or chromosomal abnormality has been identified for GLH; however, the findings reported by various research groups suggest an autosomal recessive pattern of inheritance [43, 66, 181]. Erzin et al. [50] report the only case of GLH with schizophrenia. Consanguinity has been described in three cases [34, 43, 66].

Rhombencephalosynapsis

Rhombencephalosynapsis is a rare midline brain malformation that involves the absence of cerebellar vermis, fusion (continuity) of the cerebellar hemispheres, and fusion of the dentate nuclei [216]. Rhombencephalosynapsis can occur in isolation or in combination with other anomalies such as Gómez-López-Hernández syndrome (see above), VACTERL [vertebral anomalies (V), anal atresia (A), cardiovascular defects (C), esophageal atresia and/or tracheoesophageal fistula (TE), and renal (R) and limb/radial (L)] features, and holoprosencephaly [84, 216, 219]. Individuals suffer from truncal ataxia, limb ataxia, head stereotypies, delayed motor development, abnormal eye movements, and other features are determined by supratentorial abnormalities [25].

According to some authors, only 30–35 cases of rhombencephalosynapsis have been identified in the literature from 1914 to 1995 [189, 219], but there may be over 100 cases worldwide [148]. Several studies from the United States [216, 230], Japan [219], India [148], and Turkey [12, 189] have been published. Most of these studies are case reports describing one or two cases; however, Tully et al. [216] describe their comprehensive search for patients with rhombencephalosynapsis in a database of more than 6800 individuals with brain malformations and other developmental brain disorders. The authors found and described 53 cases of rhombencephalosynapsis and the features of GLH, VACTERL, or other malformations that presented in conjunction with rhombencephalosynapsis. Based on an examination of MRI scans of 3000 children, Sener [189] 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 [12].

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 brainstem [195]. In most cases, the posterior fossa is small, resulting in downward displacement of the cerebellum and lower medulla together or cerebellum alone into the spinal canal [183]. 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 [1, 63, 68]. Chiari Type II malformations (CMII) are usually identified at or before birth [32], but may go undetected if symptoms are not apparent. This is often the case for Chiari Type I malformations (CMI), which is frequently asymptomatic and may not be recognized until adolescence or adulthood [132]. The average age of a CMI diagnosis has been reported to be 24.9 ± 15.8 years [122].

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 at the population level [92, 185]. Nevertheless, these studies are valuable in describing the epidemiology of Chiari malformations. For example, Meadows et al. [121] conducted a retrospective examination of more than 22,000 brain magnetic resonance images in the United States and estimated the prevalence of CMI at 7.8 per 10,000. Horn et al. [78] examined the 2003–2012 data collected and maintained by the Nationwide Inpatient Sample (NIS) in the United States, and found 305,726 cases of CMI, 119,632 cases of CMII, 15,540 cases of CMIII, and 79,663 cases CMIV are recorded in the database. The earlier studies from Western countries reported prevalence estimates of 8.2–8.4 per 100,000 [28, 29]. 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 [46].

Lee et al. [106] 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 3–27 months, 9 were 3–4 years of age, and 41 were 5–17 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 et al. [177] 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. Sakushima et al. also reported that Chiari malformation was more common in children than adults. In Italy, Ciaramitaro et al. [36] examined the 2011 data from the Interregional Piemonte and Valle d'Aosta Rare Disease Registry and estimated the period prevalence of CMI to be 7.74 per 100,000, which was higher for females than males.

A few studies examined incidence of Chiari malformations. In one study, 3 years of ultrasound examinations for 22,500 pregnant women from East Azerbaijan in Iran were reviewed to estimate incidence of these conditions [62]. Of the 22,500 pregnancies, 112 (or 0.5%) of fetuses had CNS anomalies and 41 had Chiari malformations. Ghavami and Abedinzadeh [62] concluded that Chiari malformations and hydrocephalus were the two most common CNS abnormalities in East Azerbaijan. O'Reilly and Torreggiani [135] scanned a sample of 147 individuals (aged 15–93 years) over a 24-month period in Ireland and calculated an incidental rate of 2% for asymptomatic CMI. Incidence was estimated at 3.08 per 100,000 in the Republic of Tatarstan in Russia, but was significantly higher in the northern (due to a high prevalence of 413 per 100,000 in the northern Baltasy region) than the southern districts of Tatarstan [23] (see Table 4).

Although the exact cause of Chiari malformation is unknown, research suggests that genetic factors are the most likely. Schanker et al. [185] 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. Birth injuries, heavy birth-weight babies, and history related to minor head or neck trauma have also been implicated in the development of Chiari malformations [76]. CMI has been found to coexist with ASD, but CMI is often under recognized in individuals with ASD because symptoms are attributed to autism [87].

Tectocerebellar Dysraphia

Tectocerebellar dysraphia is an extremely rare congenital malformation characterized by 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 to it [103]. Very few cases of tectocerebellar dysraphia have been reported in the scientific literature [212]. Children with tectocerebellar dysraphia generally have very low intellectual functioning and 40–75% die before their first birthday, largely because of hydrocephalus [145].

Tectocerebellar dysraphia is a condition so rare that prevalence and incidence estimates cannot and have not been made. We located eight reports that described the following ten cases: one 3-month-old boy in India [4]; one 5-month-old girl in Turkey [11]; two cases (one 8-year-old boy and one 2-month-old boy) but the authors also noted three other cases previously described in Switzerland [58]; one infant female in Brazil [67]; one 7-month-old male in India [103]; one 5-day-old boy in Saudi Arabia [131]; one 4-year-old girl in the United States [157]; and four cases (three girls and one boy) in Saudi Arabia [35]. Variants of tectocerebellar dysraphia (e.g., tectocerebellar dysraphism with an occipital encephalocele) may be considered structural manifestations of Joubert syndrome [157].

Other

Cerebellitis

Acute cerebellitis is an inflammatory syndrome characterized by cerebellar dysfunction ([18]; as cited in [59]). Vomiting, headaches, tremors, nystagmus, dysarthria, and states of consciousness ranging from sleepiness to coma are common symptoms of severe cerebellitis [26, 59]. Patients with acute cerebellitis may also exhibit broad-based gait disturbance, poor coordination of finger-to-nose movements (dysmetria), and irritability [26]. Cerebellitis typically occurs in early childhood during or after infection, 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 [9, 26]. In a retrospective study using 10 years of data (October 2003–June 2013) from Bambino Gensu Hospital, Rome, Italy, Bozzola et al. [26] found that 48 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).

The majority of the literature describes isolated case reports of the most severe but rare cases of cerebellitis (cf. [44]), 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 [44] for a review). Hackett et al. [70] 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 et al. [75] 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 et al. [118] found two cases of cerebellitis (1.03%). Uchizono et al. [217] reported what appears to 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 et al. [235] 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 postinfectious ataxia [193] and opsoclonus–myoclonus syndrome [205].

Cerebellar Stroke

Cerebellar stroke is characterized by complaints of dizziness, vertigo, and vomiting [232]. Pontine compression and acute hydrocephalus secondary to the obstruction of the fourth ventricle may occur because 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 [30]). The overall incidence of cerebellar stroke across all ages has been estimated to be about 1.5% of all strokes (see [94]). Researchers posit that the prevalence of cerebellar stroke is underestimated because it presents differently than more common types of stroke and the condition may be overlooked entirely or misdiagnosed as another condition [140, 182], because symptoms, such as ataxia, cannot be clearly observed during bedside examinations [232].

As is true of other cerebellar disorders, cerebellar stroke is unusual in children (see [110]). When cerebellar infarction does occur, it is documented in the research literature (see Table 5). Lin et al. [110] 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 et al. [60] found that one child developed cerebellar infarction. Thakkar et al. [208] reported that of the 120 cases of acute ataxia that occurred in children (0–18 years of age) seen at Children's Hospital of Pittsburgh between January 2003 and December 2013, cerebellar stroke was identified in 2 (1.7%) cases (see Table 5).

Risk factors for cerebellar stroke typically include trauma, drugs, CNS infection (see [74]), and inhalation of toluene-mixed paint [147]. Cases are emerging in the literature linking the coronavirus disease 2019 (COVID-19), caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), to cerebrovascular accidents, including cerebellar stroke [140, 159]. When trauma is sustained through sport, stroke may occur in boys 6.6 times more than in girls [74] and may occur with sudden movement [93]. 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 et al. [74] reported a high

Table 5 Prevalence, incidence, and/or number of cases reported in studies of investigating other cerebellar conditions

	Study details				Population					
Disorder First author (date)	Research design	Country/ region	Study period	Data source	Age (years)	Sex	*N*	Prevalence	Incidence	*N* of cases or families
Cerebellitis										
Hackett [70]	Case report	Ireland	–	–	6	F	1	–	–	1 case associated with influenza A
Hashemi [75]	Case report	The United States	–	–	9	M	1	–	–	1 case associated with *plasmodium falciparum* infection
Mazur-Melewska [118]	Retrospective evaluation, case report	Poland	2010–2015	University of Medical Sciences	5.1	84 F; 110 M	194 with Epstein-Bar virus	–	–	2 (1.03%)
Uchizono [217]	Case report	Japan	–	–	7	F	–	–	–	1 case associated with group A streptococcal infection
Xu [235]	Case report	China	–	Third Hospital, HeBei Medical University	15	2 M	–	–	–	2 cases likely associated with a viral infection
Cerebellar stroke										
Garbash [60]	Retrospective	The United Kingdom (England, Wales)	January 2004–December 2008	Pediatric Intensive Care Unit Audit Network (PICANet)	Children (< 16)	–	977 malaria cases	–	–	1 case of cerebellar stroke

Kawakami [93]	Case report	Japan	–	–	8	M	1	–	–	1 case associated with trauma during sport
Khair [94]	Case report	Qatar	–	–	4.5	F	1	–	1.5%	1 case, premature birth
Lin [110]	Case report	Taiwan	–	–	12	M	1	–	–	1
Park [147]	Case report	Korea	–	Emergency Medicine, Uijeongbu St. Mary's Hospital	57	F	1	–	–	1 case associated with toluene
Quenzer [159]	Case report	The United States	–	UC San Diego Medical Center	32	M	1	–	–	1 case linked to COVID-19 infection
Thakkar [208]	Retrospective	The United States (Pittsburgh)	January 2003–December 2013	–	0–18	–	120	1.7% among cases of ataxia	–	2
Thoon and Chan [209]	Case report	Chinese-Thai	–	–	10	F	1	–	–	1 case associated with influenza
Vafaeeshahi [220]	Case report	Iran	January 2017	Pediatric Emergency Room, Moosavi Hospital	9	F	1	–	–	1 case of cerebellar stroke

Notes: *F* female, *M* male

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 [80]. Thoon and Chan [209] 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 [30, 94]. Khair et al. [94] described a case of a 4.5-year-old girl, who was one member of a quadruplet born at 28 weeks gestation, presenting with 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 [30]. It is important to note, however, that cerebellar stroke in children remains unexplained in many cases [142, 171] as was the case of a 9-year-old girl in Iran [220].

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 health-care systems in various parts of the world (see [124]), 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 [184]. 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 [173]). 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 [173]). Thus, case studies become a very important means with which to communicate the various signs, symptoms, comorbidities, and complications associated with 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 to provide 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 a unique experimental situation for 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 molecular changes in adult Bergmann fibers required for migration and ultimate synaptic integration of the former, although this integration is not complete because the full contingent of efferent projections fails 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 the 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 are 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 believed 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 century by researchers working on brain development [1–3], or interested in 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 a 95-year old. Brody [5] concluded, based on volume shrinkage determinations correlated with cell counts, that from the age of 21 onward, there is 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 the 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 is virtually no loss of neurons before the age of 60, and even then the progress of neuron death was slow and uneven across 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 the loss of spines and the reduced 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 [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 regarding the recent history of morphological plasticity see [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 the possibility that even mild neuronal proliferation occurs 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 in 1912 [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 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, 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 neurogenesis in the adult mammalian brain is just a vestige of the phylogenetic evolution or plays an important physiological role. Experiments carried out in the two centers where adult neurogenesis is more prominent, 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 exerts an important function, closely correlated with a respective increase or decrease in either spatial (dentate gyrus) [27] or olfactory (olfactory bulb) memory [28].

Neuronal Transplantation

Neuronal Replacement in "Point-to-Point" Cerebellar Circuits

It is first important to remember that in addition to the majority of circuits, which are wired in a "point-to-point" manner as the cerebellum, the brain also has a second class of systems, called "global" [29]. The latter comprise 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 must 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's 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 (see Section "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 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 in which to test the reparative ability of grafts of embryonic neurons in "point-to-point" systems. The selected model for heredodegenerative ataxia was a 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 by using as host cerebella *nervous (nr/nr)* [37] and *Lurcher* (*Lc/+*) mutant mice [38], as well as exhibiting degeneration of PCs although these mutations involve completely different genes. Since the results were similar in each mutant, we concluded 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 at P16–P18 that some PCs start degenerating. Three to 4 weeks later, more than 99% of this neuronal population 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]. Degeneration, followed by apoptotic death (see [40]), led to a severe ataxia, which started when the homozygous mice were 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, such 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 a substantial retrograde death of granule cells [44]. In 1-year-old *pcd/pcd* cerebella, basket cell axons also seemed severely reduced 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 50–60-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, or small pieces (less than 1 mm^3) of E12 cerebellar anlagen, which 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 to 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

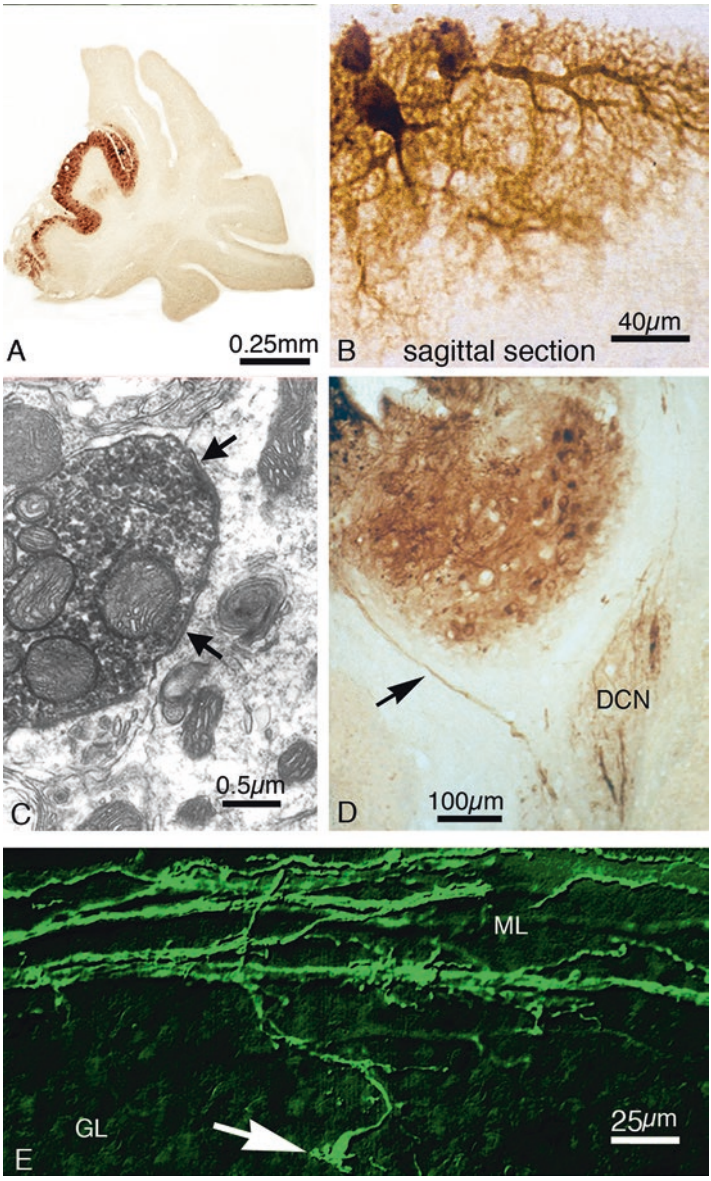

Fig. 1 Micrographs of the cerebellum of adult *pcd/pcd* mice immunostained for calbindin 2–3 months after grafting. (**a**) Low magnification of the grafted PCs that have migrated into the host cortex. The asterisk marks the graft remnant. (**b**) Sagittal section through the vermal cortex, illustrating 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)

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 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 synapsed on thorns emerging from thicker dendrites (Fig. 2a), as in the 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, despite both elements being able to establish synaptic connections (Fig. 2b). This synaptic abnormality was reliably observed for all ectopic PCs studied whatever the mutation or the situation analyzed: *weaver* and *reeler* cerebella [48, 49], transgenic mice with a plexin B2 knockout [50]. This led to the conclusion that the abnormality was not due to 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 "pinceaux" formation. Therefore, from a morphological viewpoint, despite the important synaptic failures reported above, it was concluded that the grafted PCs were synaptically integrated into the cortical circuit of the host cerebellum, and that the target-deprived host axons could recapitulate their developmental synaptic affinity and regain their 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 Section "The difficulties in restoring the corticonuclear projections argue against the possibility of successful complete PC replacement by embryonic cerebellar transplants").

Electrophysiological Results

In collaboration with Francis Crépel 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 described above. Nevertheless, all grafted Purkinje cells responded to electrical white matter stimulation with a typical all-or-none CF (complex spike) response, or complex spike followed by simple spikes. These disynaptic responses (MF–PF activation) were less frequently observed because the large amplitude and duration of the CF responses together with their short latency, usually masked the eventual consecutive excitatory

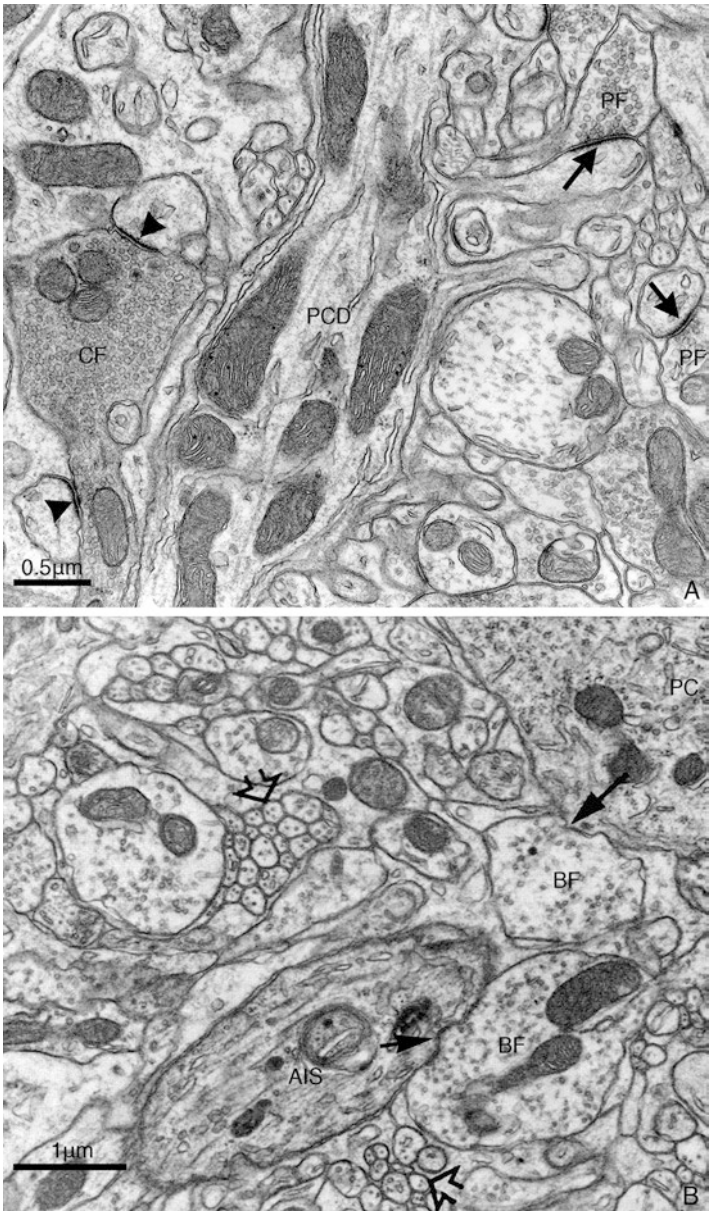

Fig. 2 Electron micrographs illustrating the synaptic input of grafted PCs. (**a**) Parallel fibers (PF) synapsing on spines (arrows) of a PC dendrite (PCD). A nearby climbing fiber (CF) synapses on two other PC spines (arrowheads). (**b**) Initial segment of the axon (AIS) of an ectopically located 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

postsynaptic potentials via MF and PFs. Finally, inhibitory postsynaptic potentials such as normally connected PCs were also recorded, corroborating the integration of the grafted neurons in the circuitry of the cerebellar cortex of the host [47].

Parasagittal Compartmentation of Grafted PCs

One of the special features of PCs is their biochemical heterogeneity, underlining the subdivision of the cerebellum into 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 are present in all PCs in adulthood [52], maintains the same topography later in development when the selective markers of adult heterogeneity begin to be expressed [53]. This continuity reflects the role of PCs as prime organizers 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 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 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, alternating zebrin+ and zebrin− PC clumps were detected. In the graft remnant, the alternating clumps 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 or size with the host stripes.

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 the result of the presynaptic inputs they received, particularly from the host olivocerebellar projection. This could 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 synaptogenesis between PCs and CFs or transiently with MFs [57]. To this end, 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 therefore able to mature without being exposed to CFs and/or MFs. In both types of transplants, alternating clusters of zebrin-1+ and zebrin-1− PCs developed without the influence of either CFs or MFs, pointing to the intrinsic nature of the biochemical heterogeneity of

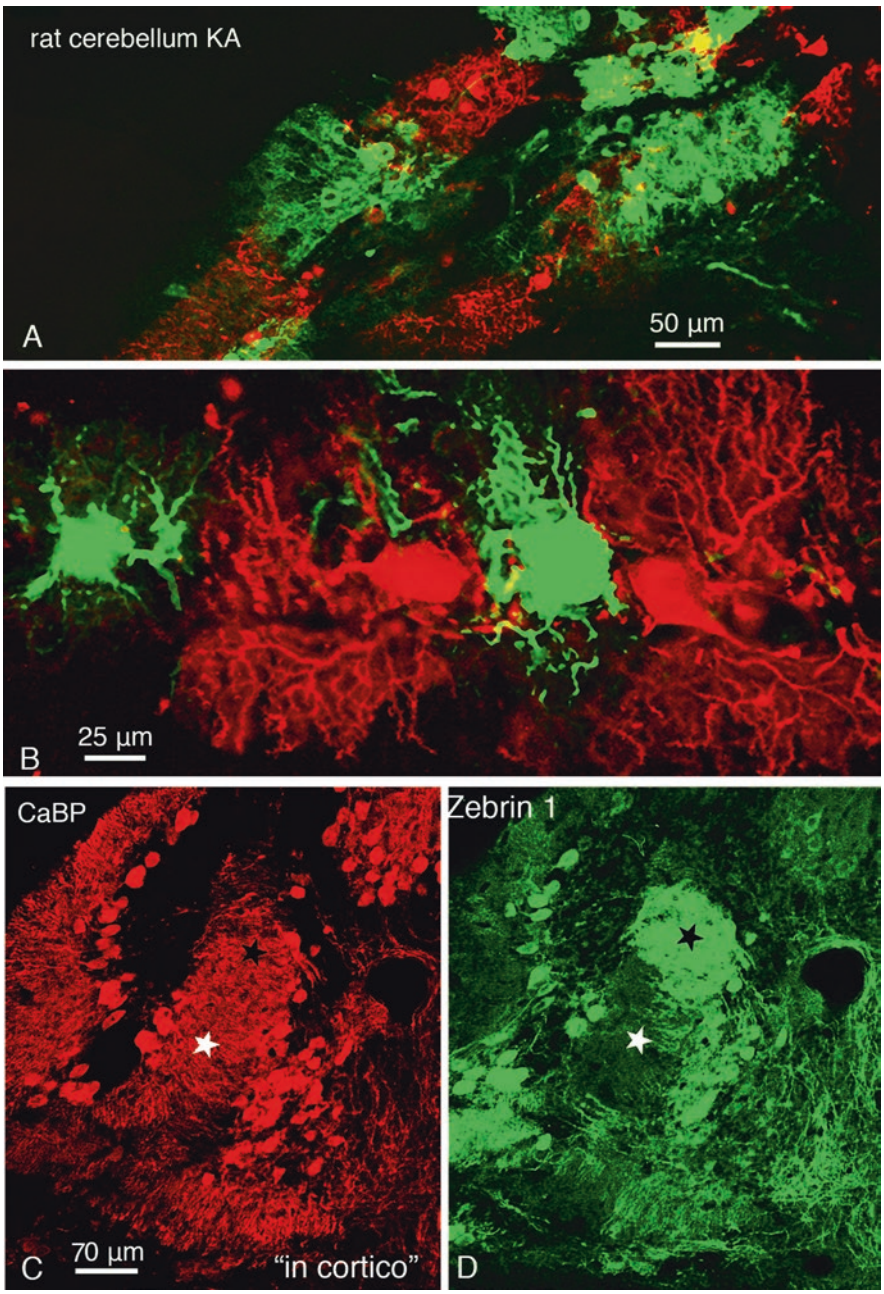

Fig. 3 (**a**, **b**) Double immunofluorescence of a grafted rat E13 cerebellar anlage into a cavity in adult rat cerebral cortex 2 months after grafting ("in cortico"). Note that while all PCs are calbindin positive (rhodamine), only part of them are zebrin-1 positive (fluorescein), revealing alternative clusters of zebrin-1 negative and positive PCs. (**c**, **d**) Two color stain of graft, derived from cell suspensions of rat E15 cerebellar anlage 1 month after injection into the PC devoid region of the kainic acid lesioned adult cerebellum, illustrating the alternating clusters of zebrin-1 positive PCs (green color) and zebrin-1 negative PCs (red color). (**c**) Graft remnant and integration of PCs into the adjacent host molecular layer. (**d**) Zone far away from the graft remnant where individual PCs alternate in the interfolial space

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.

The Difficulties in Restoring the Corticonuclear Projections Argue Against the Possibility of Successful 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, which cannot establish proper connections with their remote 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 transit the nonpermissive territory offered by the underlying granular layer, a barrier missing during the developmental period during which these axons normally reach the prospective white matter on 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 arrived 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, were in continuity with underlying clusters of grafted embryonic bridges. In these situations, their axons could grow through the bridges into the white matter and reach the DCN. These observations provided evidence emphasizing that distance was not the main obstacle for the correct growth of PC axons but rather the presence of the nonpermissive environment of the host granular layer. 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 a new corticonuclear projection in this way [58]. Although technically effective, the obtained yield was very poor, since few grafted PCs cortically integrated found their way to the DCN through the bridge.

Interestingly, the problem disappears 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 IVth ventricle of E16 rat embryos. In this case, although the surviving 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 their normal terminal domains in the DCN. However, as 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 by P8 all surviving grafted PCs remain ectopic. These results emphasize the obstacle provided by the mature granular layer to the growth of PC axons, and the difficulties confronting cerebellar grafting as a way to treat ataxia due to difficulties encountered in the mature cerebellum.

Embryonic and Adult Cells Interactions

Despite the indisputable importance of genetic programs for the developmental project of the cerebellum, it is well known that epigenetic factors that accompany cell–cell interactions are also important. In normal instances, these interactions occur between isochronic cells. Nevertheless, in reparative processes as well as during the 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 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 funneled into this stream (Fig. 4a) covering large distances, as already discussed up to two and a half folia. In preparations immunostained with anti-CaBP antibodies, subpial bipolar PCs were tangentially oriented (tangential migration) (Fig. 4b), but 2 days later they changed direction to migrate radially and penetrated 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 the relatively thick stems of Bergmann glia.

Between 11 and 14 DAG, synaptogenesis between grafted PCs and adult host presynaptic axons 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 electrophysiological results complemented 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 are

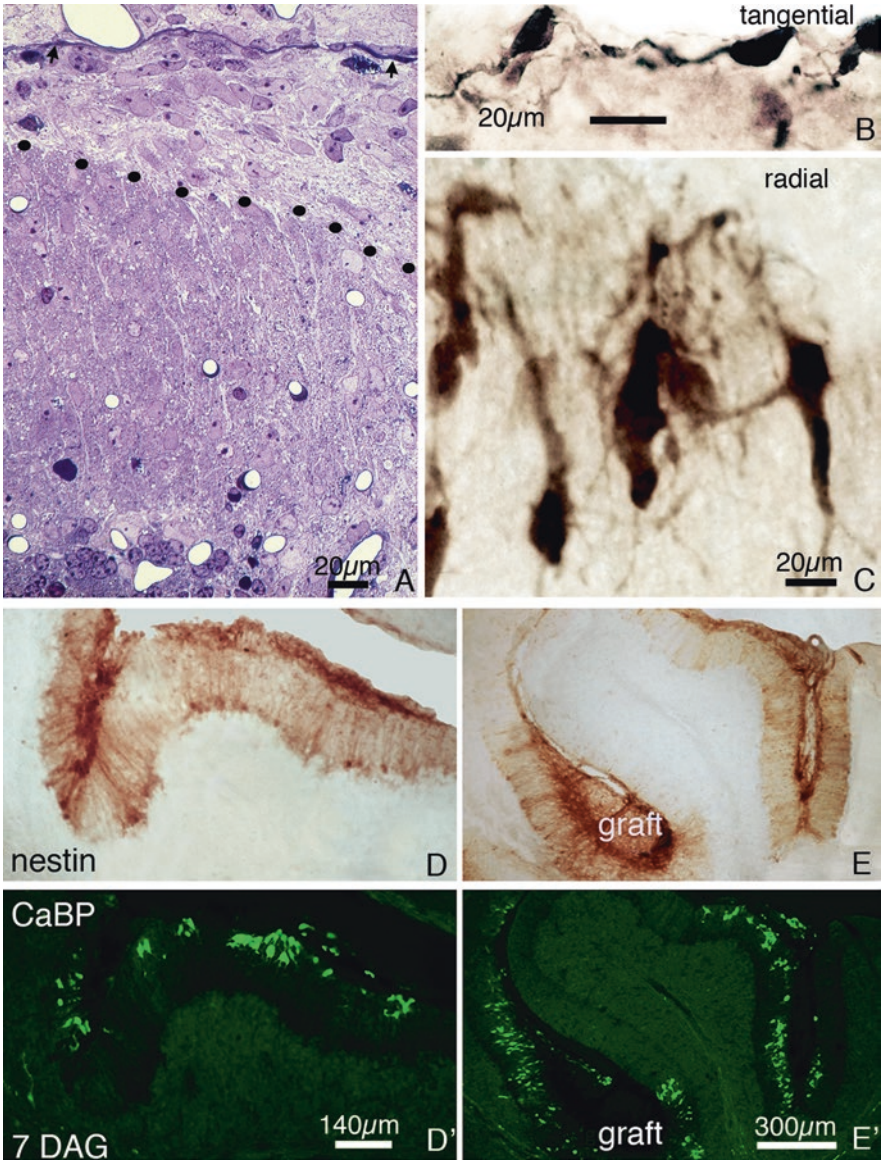

Fig. 4 Development of reciprocal graft–host interactions during PC replacement. Events taking place during the fifth to the eighth day after grafting. (**a**) Between the subpial basal lamina (marked by two arrows) and the glial limiting membrane (marked by large dots), a funneling stream of tangentially 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**, **e**') Histofluorescent visualization of grafted PCs in adult *pcd/pcd* cerebellum. 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

immature. The grafted PCs also went through a transient period of polyinnervation with an average of three distinct CFs synapsing on each PC by 10 DAG, until at least 14 DAG by which point they became monoinnervated [61].

In conclusion, the establishment of the newly formed connections mimicked very closely the time-course and sequence of events in normal development. It appears the embryonic PCs impose a program defined by their own internal clock that leads to their timely ordered synaptic integration [60]. We were fascinated by these new cyto-sociological rules imposed by the embryonic neural cells, and wanted to uncover some of the molecular mechanisms governing these embryonic/adult interactions.

Molecular Mechanisms Underlying the Interactions Between Adult Host Bergmann Fibers and Grafted Embryonic PCs Migration, a New Type of Neural Plasticity Which is Called "Adaptive Rejuvenation"

PC migration is a glial-guided migration from the ventricular cerebellar neuroepithelium to the presumptive cerebellar cortex, to form the so-called 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 to whether the grafted embryonic PCs induce adult host cells, especially the Bergmann glia, to transiently reexpress the molecular cues needed for their migration and synaptic integration into the host [60], or whether isochronic embryonic astrocytes leave the graft, acquire within the host parenchyma the radial glia phenotype, and thereby 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, where β-galactosidase activity in the cerebellum is detected exclusively on Golgi epithelial cells and their Bergmann fibers, allowing grafted cells to be distinguished from host. Presumptive molecular changes in host Bergmann fibers were investigated by immunohistochemistry with rat mAb-401 antibody (gift Susan Hockfield) [65], which identifies nestin [66], an intermediate filament protein expressed transiently by neural progenitor cells and their glial axes for neuronal migration. This antibody is therefore temporally 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 once migration was complete (13 DAG). First, no X-gal positive cells were found outside the solid graft remnant, compelling evidence

against comigration. Second, in the folia invaded by grafted PCs, and only during the short period of their radial migration, Bergmann fibers subserving this migration expressed nestin (\hvb' v. 4d, d', d, e'). This close spatiotemporal correlation allowed us to conclude that a new class of plasticity did occur, a process of "rejuvenation" induced by the grafted embryonic PCs in their adult partners. Therefore, after transient expression of molecular cues associated with PCs migration, adult Bergmann fibers become able to recapitulate the mechanisms employed in normal ontogenesis, enabling migration and synaptic integration leading to the partial 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 scarcity of adult neurogenesis but mainly because central neurons are unable to regenerate their axons, contrary to what happens in nonmammalian species [67–69] and in mammalian peripheral axons [3]. From the beginning, researchers wanted to know whether the distinct behavior of the mammalian central and peripheral neurons was intrinsic or the result of the different molecular and cellular environments they encountered. Francisco Tello [70], who worked in Ramón y Cajal's laboratory, was among the first to answer this question correctly. 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 cones-like structures at the distal ends of their proximal stumps that grew for long distances, penetrating deeply into the grafted aneural nerve fragments. For Ramón y Cajal, these observations clearly indicated that simply by providing a suitable environment, a central axon could regenerate just as a peripheral one. As a result, the study of nonpermissive molecules preventing central axons regeneration has been a research topic for the past 30 years. Extracellular matrix proteins at the glial scar (cytotactin/tenascin and proteoglycans (see in [71]), and myelin remnants [72]) have been the focus of this search. Furthermore, these studies showed that regenerative failure was not solely the result of environmental growth inhibitory molecules but also of central neurons' intrinsic properties. Indeed, after the transitional period of development, central neurons lose their ability to reset the set of genes required for axon growth. For these reasons, it is commonly accepted nowadays that the success of the regenerative process depends on the interplay between environmental cues and intrinsic properties of the damaged neurons. It is obvious that both elements should be taken into account when designing therapeutic strategies to promote 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 does not respond to axotomy with either somatic, retrograde, degenerative changes (chromatolysis), even when the injury is close to the axon hillock, as can happen in a few PCs after transection of cerebellar folia separating the anterior from the posterior vermal lobes, or dendritic changes [73]. The absence of chromatolysis was most probably responsible for the lack of regeneration observed in these neurons, because degenerative events seemed needed to activate the 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 yield "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, thin and short terminal sprouts appeared, growing slowly up until 18 months, the longest survival time analyzed [76]. After 18 months, there were numerous sprouts and were 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 cellular and molecular changes occurring in the glial scar. Activated macrophages disappeared much sooner than the initiation of sprouting. Myelin and its associated neurite growth inhibitory molecules began to decrease 3 months after the lesion. More importantly, some of the reactive astrocytes started to express Polysialylated-neural cell adhesion molecule (PSA-NCAM), the embryonic form of the neural cell adhesion molecule, at this time the nonpermissive nature of the early glial scar changing completely into a permissive substratum for neurite outgrowth. Therefore, the belated axon growth attempt takes the form of early thickness increase and late terminal sprouting, the latter occurring at the same time as changes in the glial scar. This almost exclusive response of PCs to axotomy confers these neurons the reputation as the central neurons with the poorest spontaneous regenerative capacity.

The behavior of inferior olivary neurons, also axotomized at their distal arbors – CFs – after folial 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 are known for 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 information suggested that during development, young postmitotic neurons have 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

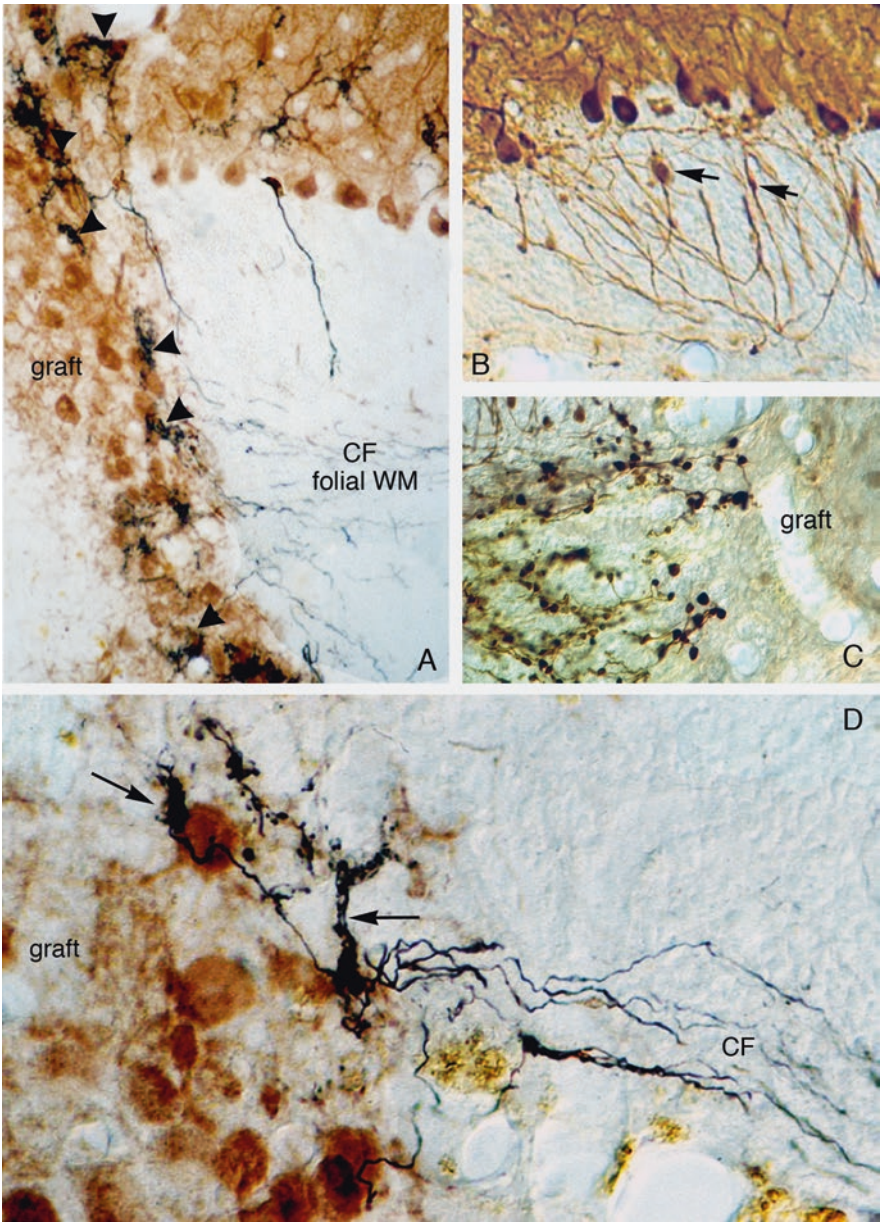

Fig. 5 Axotomy and transplantation, a combine approach in adult rat cerebellum to assess the intrinsic capacity of adult neurons to regenerate in permissive environment. After a cut separating anterior for posterior vermal cortices, the lesion site is filled with rat E13 cerebellar anlage. Survival times 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.

(continued)

(**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)
Asterisk: The micrographs illustrated this chapter have been adapted from personal publications in the topic. (See Refs. [29, 46, 55, 56, 58, 60, 62, 64, 77])

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 has been used extensively in cerebellar lesions.

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 by using this approach, including 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 till 12 months to allow for the study of the late sprouting of PC axons [85]. In all cases, the presence of a grafted permissive growth substrate allowed CFs to regenerate into the grafts, although the newly formed terminal arbors were quite different for each type of graft. Only when the graft was embryonic cerebellum, their normal target, the regenerative branches, were able to form their characteristic CFs on the dendritic trees of grafted PCs (as illustrated in Fig. 5a, 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, transplants have 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 Approach

Origins of the Cells: Embryonic and Adult Multipotent Stem Cells and Immortalized Cell Lines

Although the recent discovery of neural stem cells in the CNS of adult mammals has changed our vision of a static adult CNS, it did not 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 from 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 optimism 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 naïve stem cells 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 for their abundance in such classes 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, including bone marrow-derived mesenchymal stem cells [89] and even immortalized multipotent neural cell lines generated via retrovirus-mediated v-*myc* transfection [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 were 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] in the cerebellar white matter. Two important features allowed for their identification: (1) the expression of the stem cell marker prominin-1 and (2) the lack of neuronal and glial cell lineage 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 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 forebrain and cerebellum-derived neurospheres give rise to neurons resembling those found endogenously in the brain. In other words, neural stem

cells progeny have regional characteristics and are not totipotent since they have already undergone 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 from somatic differentiated cells (a tadpole intestinal cell), demonstrated that, when transplanted into an enucleated egg, the nucleus is reprogrammed to a pluripotent stage and may give rise to a 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 is reversible, thanks to a new mechanism named reprogramming. Reprogramming implies that fully differentiated cells could dedifferentiate and somatic cells transform into pluripotent stem cells through the inductive action of suitable transcription factors, and then differentiate again, but this time into the desired cell class [96]. Shinya Yamanaka's team publication [96], showing that expression of 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 new opportunities offered by reprogramming, 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 the building of in vitro models of genetic and acquired cerebellar diseases, providing superior material to study molecular and cellular pathomechanisms of the precise pathways leading to cerebellar degeneration. However, this, as well as the potential of such models for drug screening, is totally outside the scope of 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. The derived stem cells were able to be transformed in turn into 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 into unlimited categories of differentiated cells, especially the countless classes of central neurons. Also of great interest is the possibility to reprogram human fibroblasts directly into neurons, without passing through the status of a multipotent neural stem cell. Indeed, by 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 ability to establish functional synaptic connections [99].

Regarding the cerebellum (i.e., 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 of 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 rather enhanced reprogramming efficiency two- to threefold. On the contrary, when Zic3 is blocked by shRNA-mediated knockdown of endogenous Zic3 during iPSC generation, the reprogramming efficiency decreases [100].

Cerebellar Transplantation of Granule Cells

Transplantation of external granular layer cells was 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 into the early postnatal cerebellum [103, 104]. Nevertheless, and despite the existence of excellent murine models of ataxia subsequent 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 an 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 resulted in their engraftment, followed by their differentiation 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 putative efferent fibers of the grafted cells, were not considered in this study [103].

Naïve stem cells of two different origins (cerebellar-derived multipotent astrocytic stem cells and embryonic stem cell-derived neural precursors [107]) have been used in the *weaver* mutant mouse, another model of an agranular cerebellum [47]. Neither of them yielded neurons with a 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 the 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 into human induced cerebellar granular-like cells (hiGCs) [109] by the combination of three transcription factors (Ascl1, Sox2, and Oct4), followed by treatment with three secreted factors (BMP4, Wnt3a, and FGF8b). This protocol is a direct shortcut to convert one adult cell phenotype into a totally different one, without passing through a multipotent stem cell state. The hiGCs were used to assess their ability to replace missing cells in the cerebellum of $Nmyc^{TRE/TRE}$:tTS, a Nmyc conditional knockout mouse [110], a mutation characterized by severe microencephaly, including a profound atrophy of about 65% of the cerebellar mass affecting mainly 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. While the results with the former were obtained after newborn transplantations, those of the third model of an agranular cerebellum were obtained with grafts from 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 that 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 naïve 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. This was done either by transfecting with viral vectors the genes coding for transcription factors involved in the 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, 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 a 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 cyclopamide. This inhibition prevented the expression of Atoh1 and the formation of granule cells [116]. Finally, in 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 coculture 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 cocultured 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 plate. One month after transplantation, the surviving grafted cells appeared as normally polarized PCs, located at the interface of the molecular and granular layers, and with complete afferent and efferent synaptic integration. Indeed, their axons crossed the granular layer to enter the white matter axis, and some of them even reached their terminal domains, and established synaptic connections with DCN neurons. Therefore, the PCs derived from embryonic stem cells behaved in the same way as PC progenitors transplanted in embryonic cerebellum. By using 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 questionable clinical potential as already discussed for PCs after E12 cerebellar grafts into adult *pcd/pcd* mutants (see Section "The difficulties in restoring the corticonuclear projections argue against the possibility of successful 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.

Future Prospects

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 will the physiological mechanisms of regeneration available to the brain. The approach of genetic activation of neurogenesis in adult injured brains, particularly by transformation of reactive astrocytes into neurons able to replace the missing ones, leaving 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 should be considered from now on as the same as other body tissues. Although no one knows what the future holds, these current results give us great hope in a brighter future for reparative neurology.

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