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STATE-OF-THE-ART REVIEW

Regulation of early cerebellar development

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The study of cerebellar development has been at the forefront of neuroscience since the pioneering work of Wilhelm His Sr., Santiago Ramón y Cajal and many others since the 19th century. They laid the foundation to identify the circuitry of the cerebellum, already revealing its stereotypic three-layered cortex and discerning several of its neuronal components. Their work was fundamental in the acceptance of the neuron doctrine, which acknowledges the key role of individual neurons in forming the basic units of the nervous system. Increasing evidence shows that the cerebellum performs a variety of homeostatic and higher order neuronal functions beyond the mere control of motor behaviour. Over the last three decades, many studies have revealed the molecular machinery that regulates distinct aspects of cerebellar development, from the establishment of a cerebellar anlage in the posterior brain to the identification of cerebellar neuron diversity at the single cell level. In this review, we focus on summarizing our current knowledge on early cerebellar development with a particular emphasis on the molecular determinants that secure neuron specification and contribute to the diversity of cerebellar neurons.

Introduction

The mature cerebellum controls our fine motor skills and actively participates in body balance, posture, motor learning, feeding and cognition [1,2]. Cytoarchitectonically, the mature cerebellum consists of a three-layered folded cortex that surrounds a central collection of neurons known as the deep cerebellar nuclei (DCN), which are embedded in the cerebellar white matter (Fig. 1) [3,4]. The deepest cortical layer, the granule cell layer, contains small glutamatergic granule cells, which receive and process afferent information from brainstem precerebellar mossy fibre neurons [5–7]. Interspersed among the granule cells is a small subpopulation of glutamatergic interneurons, known as unipolar brush cells, which also contribute to the processing of mossy fibre input [8]. One should note that unipolar brush cells are particularly predominant in three regions of the cerebellum, namely, the nodule, uvula and flocculus [9–11]. Superficial and outer to the granule cell layer, a monolayer of large GABAergic neurons, Purkinje cells, receives and processes afferent information from brainstem precerebellar climbing fibre neurons [12,13]. The outermost cortical layer, the molecular layer, contains distinct medium size GABAergic interneurons as well as dendrites and axons from Purkinje and granule cells, respectively [14,15]. Purkinje cells project onto glutamatergic

Abbreviations
bHLH, basic helix-loop-helix; BMPs, bone morphogenetic proteins; DCN, deep cerebellar nuclei; E, embryonic day; EGL, external granule layer; GABA, gamma-aminobutyric acid; MLI, molecular layer interneuron; PC, purkinje cell; RL, rhombic lip; scRNA-seq, single cell RNA sequencing; snATAC-seq, single nucleus assay for transposase accessible chromatin; snRNA-seq, single nucleus RNA sequencing; VZ, ventricular zone.

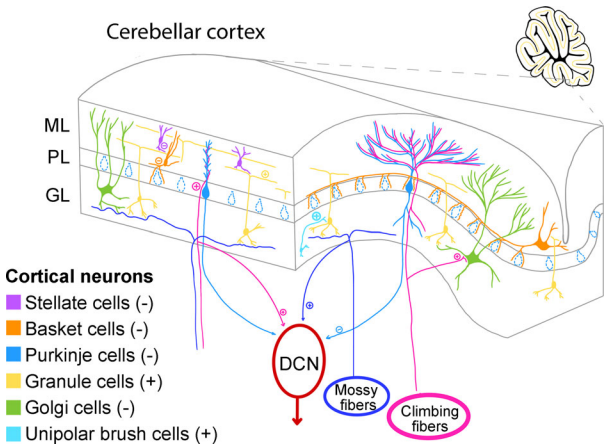

Fig. 1. Schematic display of cerebellar cortical cytoarchitecture. The cerebellar cortex is composed of three distinct cellular layers called the molecular layer (ML), Purkinje cell layer (PL) and granule cell layer (GL). Excitatory (+) and GABAergic (-) cerebellar neurons are colour-coded. Note that unipolar brush cells primarily locate in the nodule, uvula and flocculus (see the text). Afferent projection mossy fibres (from the spinal cord and brainstem), as well as climbing fibres (from the inferior olive), and the efferent cerebellar projections from the deep cerebellar nuclei (DCN) are indicated. For greater details on cerebellar afferent and efferent connectivity, please see [17,187,188].

neurons of the DCN, which represent the unique efferent system of the cerebellum [16,17]. These glutamatergic DCN neurons in turn project to diverse targets in the brain such as motor and non-motor thalamic nuclei, as well as regions involved in reward processing such as the ventral tegmental area [18].

Over the last three decades, the developmental origin of each of these cerebellar neuron types has been intensively investigated. In this review, we discuss the current knowledge of the molecular determinants that safeguard the development of each of the major cerebellar neurons (DCN neurons, granule cells, unipolar brush cells, Purkinje cells and inhibitory interneurons), with a particular focus on the transcription factors that control their specification. In the last part of our review, we summarize recent studies that apply single cell RNA-sequencing (scRNA-seq) to further elucidate the diversity that arises from cerebellar progenitors during development.

Early specification of the cerebellar territory: The cerebellar anlage

The cerebellum develops from the rostral aspect of the rhombencephalon, a region also known as the hindbrain, that locates between the developing mesencephalon (or midbrain) and the spinal cord (Fig. 2). In vertebrates, the rhombencephalon is transiently segmented into seven to eight small morphological subunits called rhombomeres, which give rise to the cerebellum, the pons and the medulla oblongata [19,20]. Some of these morphological rhombomeres can be further regionalized (into pseudomeres) according to the differential expression of a number of patterning genes [21–23]. In humans, these rhombomeres appear by embryonic day (E) 29, whereas in mice, they are recognizable at E8.5 [20]. The morphological borders of the rhombomeres coincide with the differential, and oftentimes overlapping, expression of genes belonging to the Hox superfamily of transcription factors [24–26]. For instance, the border between rhombomere 1 and 2 is characterized by the expression of *Hoxa2* in the latter and its absence in the former, whereas the border between rhombomere 2 and 3 is established by the expression of *Hoxb2* in rhombomere 3 and its absence in rhombomere 2 [27,28]. The molecular pattern of Hox gene expression is a great contributor to anterior-posterior specification of the developing hindbrain. Three different brain structures arise from the rhombencephalon: (a) the cerebellum from rhombomere 0/1, (b) the pons from rhombomeres 2-3 and (c) the medulla oblongata from rhombomeres 4 to 7/8.

One of the first key events in cerebellar development is the early establishment of the isthmic organizer at the midbrain–hindbrain boundary (Fig. 2) [29–32]. This organizer expresses diffusible morphogens that pattern the differentiation of the neighbouring nervous tissue and induce the cell fate identity of the midbrain and the cerebellum [33]. Several transcription factors are differentially expressed anterior and posterior to the isthmic organizer, which further instruct the patterning of the anterior-posterior axis of the neural tube [34]. In particular, the early expression (around E7.5 in mice) of two antagonistic transcription factors: Otx2 and Gbx2, at the anterior and posterior borders of the isthmic organizer, determine the rostral and caudal regions of the developing central nervous system, respectively (Fig. 2) [35–42]. In mice, the ablation of *Otx2*, and its close family member *Otx1*, results in the loss of midbrain structures, which in turn transforms them into cerebellar-like regions [43–45]. Conversely, the misexpression of *Gbx2* alters the correct development of the cerebellum and the positioning of the isthmic organizer [42,43,46–48].

The major morphogens emanating from the isthmic organizer are Wnt family members (Wnt) and fibroblast growth factor (Fgf) ligands (Fig. 2B). In the early 1990s, two independent groups discovered the essential function of the *int-1* (now called *Wnt1*) gene in the correct development of the midbrain and the

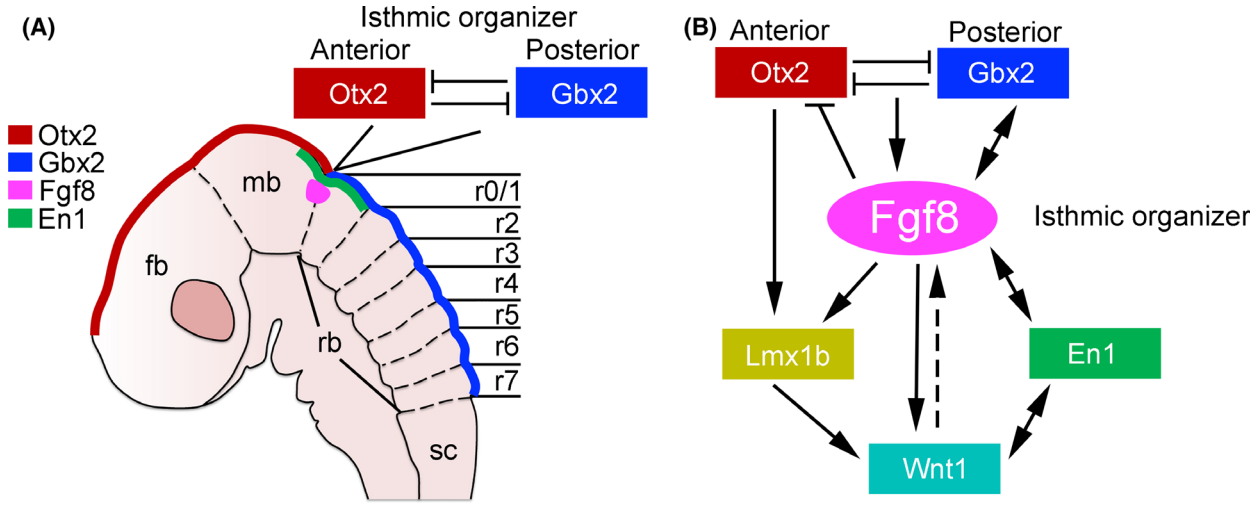

Fig. 2. Establishment of the mid/hindbrain border. (A) Schematic display of the molecular and anatomical establishment of the anterior and posterior brain borders by the expression of *Otx2* and *Gbx2*, respectively, in a developing mouse embryo between E8-E8.5. The forebrain (fb), midbrain (mb), rhomboencephalon (rb) and spinal cord (sc) are indicated. The expression of the essential factors Fgf8 and En1 for cerebellar development are illustrated (see the text). Note that the 7 to 8 transient morphological units of the rhombencephalon (rhombomere, r) are also indicated. (B) Molecular pathways operating in the midbrain–hindbrain border and the establishment of the isthmic organizer (see the text).

cerebellum, as its mutation results in the total loss of these two brain structures in mice [49,50]. A few years later, in 1996, Crossley and colleagues demonstrated the function of the diffusible factor Fgf8 in the development of midbrain and cerebellar structures [51]. Since its identification as an instructive signal, Fgf8 has received enormous attention as a key factor in patterning the midbrain and cerebellum, as it is sufficient to ectopically induce both structures in gain-of-function studies, a function that is not mimicked by Wnt1 [44,51–55]. During development, the regulation of Fgf8 expression in the isthmic organizer appears to result from the hierarchical and sequential expression of the diffusible Fgf4 ligand from the notochord, which induces expression of the *engrailed homeobox 1* (*En1*) transcription factor in the midbrain. En1 in turn activates the expression of *Fgf8* in the isthmic organizer [56]. The expression of *En1* and its close relative *En2* is critical for cerebellar development, as inactivating *En1* causes cerebellar aplasia, while the loss of *En2* causes cerebellar hypoplasia with abnormal cerebellar foliation in mice [57–59]. Two isoforms of Fgf8, Fgf8a and Fgf8b, seem to act differentially in the specification of the midbrain and the cerebellum [60,61]. Indeed, Fgf8a induces midbrain development, whilst Fgf8b induces cerebellar development [61–64]. Of note, the presence of all isoforms of Fgf8 have been demonstrated to be indispensable for the correct development of the midbrain and cerebellum [65]. Interestingly, the duration of *Fgf8* expression in the isthmic organizer seems to carry most of its instructive information, as shown by the temporal conditional ablation of *Fgf8* during development. It is now clear that the sustained expression of *Fgf8* is critical to restrict the expression of *Otx2* rostral to the isthmic organizer in order to create an Otx2 free zone in which the cerebellum can form [53,66]. Simultaneously, Fgf8 induces the expression of *Gbx2* caudal to the isthmic organizer [64]. The expression of *Gbx2* is key for proper development of the cerebellum and other neuronal derivatives from rhombomeres 1 to 3, as shown by the analysis of *Gbx2* null mutant mice. These mice completely lack the cerebellum and most other derivatives from these rostral rhombomeres. In addition to restricting the rostral expression of *Otx2* and inducing the caudal expression of *Gbx2*, Fgf8 regulates the expression of the transcription factor *LIM homeobox transcription factor 1 beta* (*Lmx1b*), which directly contributes to the maintenance and stabilization of *Wnt1* expression in the isthmic organizer [67–69]. Furthermore, the simultaneous ablation of *Lmx1b* and its closely related family member *Lmx1a* severely alters the general specification of the hindbrain, which adopts a much more posterior ("spinal cord-like") fate. *Lmx1b*/*Lmx1a* double mutant mice also lack a recognizable cerebellum [67,68,70]. Thus, the early specification of the midbrain–hindbrain border, a prerequisite for the development of a cerebellar primordium, relies on a coordinated molecular

network of several transcription factors and signalling cascades (briefly summarized in Fig. 2).

The primordium of the cerebellum, also known as the cerebellar anlage at the dorsal aspect of rhombomere 1, can be first detected at E9 in mice and has been acknowledged to arise not from anatomical but rather molecular boundaries [33,41,71]. The cerebellar anlage originates from a region in which rostral and caudal borders are limited by the expression of *Otx2* and the transcription factor *Hoxa2*, respectively. As described above, the ablation of *Otx2* results in the rostral expansion of the cerebellum [38,43–45]. In a similar manner, the ablation of *Hoxa2* causes a caudal expansion of cerebellar tissue into rhombomeres 2/3, whereas the ectopic expression of this factor in rhombomere 1 suppresses the specification of cerebellar neurons altogether [72,73]. Notably, the restriction of *Hoxa2* caudal to rhombomere 1 is also directly regulated by Fgf8 [23,74]. Thus, the creation of an *Otx2*/*Hoxa2* exclusion zone is critical for the establishment of the cerebellar anlage. As mentioned above, *Gbx2* expression is key in cerebellar development, although its function seems not to be directly involved in the differentiation of cerebellar tissue, but rather in curtailing *Otx2* expression. In support of this, work in zebrafish has demonstrated that cerebellar phenotypes resulting from *Gbx2* null mutations can be rescued by reducing *Otx2* expression [69,75]. Furthermore, the focal overexpression of *Gbx2* suffices to induce cerebellar-like tissue in the midbrain, but this phenotype appears to be the direct result of a downregulation in *Otx2* expression [40,41]. The anterior to posterior patterning of the cerebellar anlage depends on diffusible signals (Fgf8, Wnt1) from the isthmic organizer and on the differential expression of various transcription factors (*Otx2*, *Gbx2*, *Hoxa2*, *En1*/*2*). The dorsal to ventral patterning of the cerebellar anlage, and the proliferation of its progenitor cells, is primarily mediated by the diffusible morphogen Sonic hedgehog, which is expressed by cells located in the ventral midline and the floor plate of the neural tube [76–80].

The cerebellar anlage contains two distinct, adjacently located progenitor zones, called the rostral rhombic lip and ventricular zone, from which all cerebellar neurons emerge during development (Fig. 3A) [81–83]. Fate-mapping studies, histological analyses and transplantation experiments have demonstrated that the rostral rhombic lip generates all excitatory cerebellar neurons, whereas the ventricular zone is the source of GABAergic neurons. In the following sections of this review, we will focus on the molecular pathways involved in the differentiation and specification of cerebellar neurons.

The rhombic lip and the origin of excitatory cerebellar neurons

Since the turn of the 20th century, the dorsal most part of the developing rhomboencephalon has been acknowledged as the germinal zone of cerebellar and precerebellar neurons. Classically, this region is called the rhombic lip and is subdivided into a rostral rhombic lip (in r0/1) and a caudal rhombic lip (that expands from r2 to r7/8 and pseudomeres 9-11) (see above and Fig. 3A). Initial birth-dating studies revealed the rostral rhombic lip to be the bona fide neurogenic niche of all excitatory neurons that populate the mature cerebellum, whereas the caudal rhombic lip is the source of excitatory afferent mossy fibre precerebellar neurons (pontine, lateral reticular and external cuneate nuclei) [21,84–88].

The rhombic lip locates immediately after the roof plate in the neural tube and was first characterized by the expression of the basic helix-loop-helix (bHLH) transcription factor *Atonal homologue 1* (*Atoh1*, formerly known as *Math1*) (Fig. 3A) [89–96]. In the rhombencephalon and the spinal cord, the roof plate acts as a signalling centre that imposes a dorsal fate to its neighbouring neural tube progenitor cells by secreting signalling cues such as Wnt ligands and bone morphogenetic proteins (BMPs) [97]. The roof plate is known to express *Lmx1a*, the expression of which is particularly critical for the correct specification of the cerebellar excitatory neurons destined to populate the vermis (the region located at the midline of the cerebellum) [97–99]. In this context, available evidence illustrates that the double mutation of *Lmx1a*/*Lmx1b* in mice results in the absence of the roof plate and also precludes the expression of *Atoh1* in the rhombic lip [67,68,70,100,101]. A recent scRNA-seq study of the developing cerebellum revealed a novel population of cells (Fgf17+, Calb2+) that derive from the roof plate and depend on Fgf8 to pattern the cerebellar vermis via Wnt/ß-catenin and FGF/ERK signalling [102]. The rostral rhombic lip is the source of the three distinct excitatory neuronal derivatives that emerge in a stereotyped temporal order: (a) deep cerebellar nuclei (DCN) neurons (predominantly between E10.5 and E12.5), (b) external granule layer (EGL) cells (between E12.5 and birth) and (c) unipolar brush cells (between E14.5 and the first days of postnatal life, with the earliest cells populating the nodule and uvula) [9,87,103–105]. A recent study shows that the bHLH transcription factor *Oligodendrocyte factor 3* (*Olig3*) is an early determinant of cerebellar excitatory neuron specification, whose function is particularly prominent in the development of DCN neurons and early born

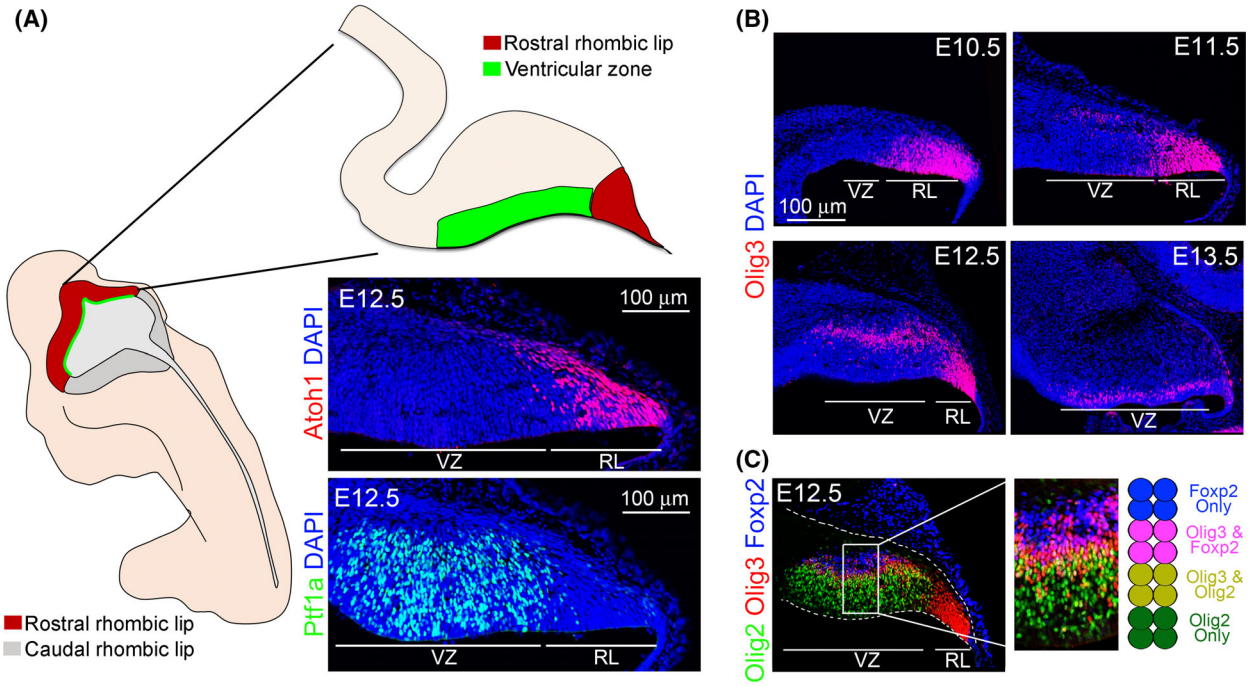

Fig. 3. Cerebellar anlage. (A) Left: schematic view of a developing mouse embryo at E10.5. The rostral and caudal rhombic lip are indicated. Top right: schematic sagittal view of the cerebellar anlage illustrating the rostral rhombic lip and the cerebellar ventricular zone. Bottom right: sagittal sections of the mouse cerebellar anlage stained with antibodies against Atoh1 (red) and Ptf1a (green) that label the rostral rhombic lip (RL) and cerebellar ventricular zone (VZ), respectively, at E12.5. The sections were counterstained with DAPI (blue). (B) Sagittal sections of the mouse cerebellar anlage stained with antibodies against Olig3 (red) at distinct developmental stages. (C) Sagittal section of the mouse cerebellar anlage stained with antibodies against Olig3 (red), Olig2 (green) and Foxp2 (blue) at E12.5. Note that Olig2 is exclusively expressed in ventricular zone progenitors, while Olig3 is transiently expressed in Foxp2+ newborn Purkinje cells. A fraction of ventricular progenitor cells co-express both Olig3 and Olig2. Photomicrographs were taken from Lowenstein et al., 2021 (ref [106]) that was published under the terms of the Creative Commons Attribution License, which permits unrestricted use and redistribution, provided that the original author and source are credited (https://creativecommons.org/licenses/by/4.0/).

EGL cells in mice (Fig. 3B) [106]. *Olig3* expression can be detected in the presumptive rostral rhombic lip as early as E9.5, a day that precedes the expression of *Atoh1* in this region [106]. A partial overlap in the expression of *Olig3* and *Atoh1* exists between E10.5 and E13.5, after which *Olig3* expression becomes downregulated. Ablation of *Olig3* severely compromises the generation of DCN neurons and results in the elimination of about half of EGL cells [106]. Complementarily, the ablation of *Atoh1* results in the almost complete elimination of EGL cells and a significant impairment in the development of DCN neurons [89,93,94,96,107–109].

In 2006, the work of Fink and colleagues carefully characterized the spatio-temporal development of DCN neurons from the rostral rhombic lip using a battery of antibodies against transcription factors characteristically expressed in excitatory neurons, such as Pax6, Tbr1 and Tbr2 [104,110–112]. In doing so, the authors found that most DCN neurons become specified around E10.5 in the rhombic lip and initiate a tangential migration forming a stream of cells under the pial surface before coalescing in a region called the nuclear transitory zone. Interestingly, during their migration to the nuclear transitory zone, DCN neurons undergo a series of transcriptional changes that might account for their maturation. First, these cells express Pax6 as they become post-mitotic and initiate their migration away from the rhombic lip; second, they initiate expression of Tbr2 as they reach the nuclear transitory zone; and finally, they downregulate their expression of Pax6 and Tbr2 while upregulating Tbr1 expression (Fig. 4A,B). The sequential expression pattern of Pax6 > Tbr2 > Tbr1 displayed by DCN neurons appears to be shared, and possibly conserved, with the development of excitatory pyramidal neurons of the cerebral cortex [113]. As cerebellar development progresses, DCN neurons become organized medial to lateral in three distinct nuclei: nucleus fastigii, nucleus interpositus and nucleus dentatus (Fig. 4C). These

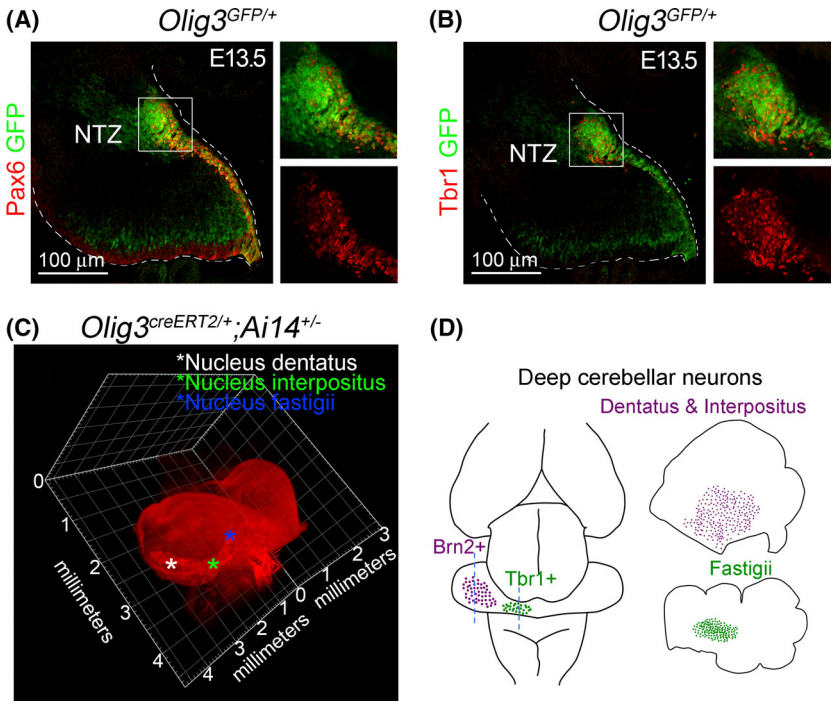

Fig. 4. Deep cerebellar nuclei neuron development. (A, B) Immunofluorescence characterization of Tbr1+ DCN neurons (B, in red), and Pax6+ EGL cells (A, in red) prepared from E13.5 *Olig3*$^{GFP/+}$ mouse embryos. The nuclear transitory zone (NTZ) is marked in A and B. All cerebellar sagittal sections were stained against GFP (green). Olig3 has been recently uncovered as a critical factor in the development of DCN neurons (see the text). (C) A sagittal three-dimensional reconstruction of a cerebellum prepared from a *Olig3*$^{creERT2/+}$; *Ai14*$^{+/-}$ mouse embryo that was recombined with tamoxifen at E10.5 to mark all cerebellar neurons with a history of Olig3 expression with tomato fluorescent protein. The mouse embryo was imaged at E19. The three known DCN nuclei are indicated with asterisks. (D) Schematic display of DCN nuclei positive for Brn2 (dentatus and interpositus) and Tbr1 (fastigii). This figure is adapted from Lowenstein et al., 2021 (ref [106]) that was published under the terms of the Creative Commons Attribution License, which permits unrestricted use and redistribution provided that the original author and source are credited (https://creativecommons.org/licenses/by/4.0/).

nuclei can be distinguished by the expression of homeobox transcription factors *Tbr1* and *Brn2*. Indeed, fastigii cells express Tbr1, while the interpositus and dentatus neurons express Brn2 (Fig. 4D) [104,106]. In addition to DCN neurons, EGL cells also depend on Pax6 for their correct development [114]. Recent evidence shows that the ablation of *Pax6* does not compromises the initial determination and migration of DCN neurons nor of late born EGL cells, but it results in massive apoptosis of these rhombic lip derivatives and the agenesis of most cells belonging to these neuronal types [114].

In the later stages of cerebellar development (after E12.5), rhombic lip precursor cells predominantly express either *Pax6* or *Tbr2*, and this seems to account for the differential generation of EGL (Pax6+) and unipolar brush cells (Tbr2+) [103]. The genesis of EGL cells occurs from E12.5 to E16.5, during which these cells form a layer immediately below the pial surface and undergo a period of prolonged clonal expansion. In general, EGL cells retain *Atoh1* expression and co-express *Pax6* in addition to the bHLH factor *Neurod1* and other zinc finger proteins such as *Insm1*, *Zic1* and *Zic3* [89,93,96,114,115]. In 2009, three independent reports illustrated the important function of *Neurod1* in the differentiation of EGL cells into their granule cell derivatives, as the deletion of this factor drastically prolongs the proliferation of EGL cells and induces apoptosis in the developing cerebellum [10,116,117]. The extended proliferation and lethality of *Neurod1*-deficient EGL cells might result from the loss of the pioneer and proneural function that *Neurod1* exerts in these cells by mediating, among other molecular cascades, the expression of different elements of the Notch signalling pathway [118–120]. In this context, available evidence shows that the expression of *Neurod1* is sufficient to drive terminal neuronal differentiation of EGL cells during both the development and postnatal life of mammals and other vertebrates [121–123]. Furthermore, recent reports show that elevated levels of *Neurod1* expression can drive medulloblastoma cells into granule cell differentiation,

demonstrating that *Neurod1* overrides the oncogenic mutations present in medulloblastoma cells [124–126].

Unipolar brush cells represent a late derivative of the rostral rhombic lip and are characterized by the expression of the transcription factor *Tbr2*, which is expressed during their determination and retained in their mature states [8,103,127]. The differential expression of *calretinin* and *mGluR1α* appears to distinguish two subtypes of unipolar brush cells termed as type I (calretinin+) and type 2 (mGluR1α+), although a recent scRNA-seq study found that many genes expressed in unipolar brush cells show continuous variation and might therefore not represent distinct cell types but a continuum [103,128–134]. In addition to migrating to the cerebellar cortex, rostral rhombic lip derived unipolar brush cells also migrate towards the cochlear nucleus in the medulla oblongata, where they integrate into the central auditory circuit [103,127,135]. As perhaps expected, the ablation of *Tbr2* completely precludes the development of all unipolar cell types without significantly interfering with the development of DCN neurons or EGL cells, illustrating the essential requirement of *Tbr2* in unipolar cell development [127].

Altogether, the temporal development of rostral rhombic lip derivatives appears to correlate with the sequential, and to a certain extent overlapping, expression of *Olig3* (early born DCN neurons), *Olig3*/*Atoh1* (late born DCN neurons), *Olig3*/*Atoh1* (early born EGL cells), *Atoh1* (late born EGL cells) and *Atoh1*/*Tbr2* (unipolar brush cells) in the progenitor pool of this neurogenic niche.

The ventricular zone and the origin of GABAergic cerebellar neurons

The ventricular zone, adjacently located to the fourth ventricle, is the unique source of all GABAergic cerebellar neurons, which can be divided in two major populations: Purkinje cells and inhibitory interneurons. Similar to the rhombic lip, the ventricular zone generates GABAergic cells in a stereotyped temporal manner, first generating Purkinje cells (between E11.5 and E13.5 in mice) and then inhibitory interneurons (from E14.5 to the first days of birth) [15,82,136,137]. The development of both GABAergic neuron types fully depends on the expression of the bHLH *Pancreas transcription factor 1a* (*Ptf1a*, Fig. 3A), the mutation of which precludes their specification and results in cerebellar agenesis in both humans and mice [109,137–139].

During the early specification of Purkinje cells, a number of bHLH factors have been reported to be co-expressed with *Ptf1a* in progenitor cells of the ventricular zone, including *Ascl1*, *Neurog1*, *Neurog2*, *Olig3* and *Olig2* (see below). Despite the fact that *Ascl1* is highly expressed in the ventricular zone during the specification of Purkinje cells, the loss of this factor has little effect on their specification, albeit the generation of cerebellar inhibitory interneurons and glial cells is largely affected [140–142]. Similarly, the ablation of *Neurog1* from ventricular zone progenitors does not significantly alter the development of Purkinje cells, but does slightly impair the development of cerebellar inhibitory neurons, with a mild reduction in the numbers of interneurons generated in early postnatal life [143]. Neurog2 actively regulates the proliferation of ventricular zone progenitor cells that generate Purkinje cells [144]. The ablation of *Neurog2* does not interfere with Purkinje cell specification but alters their postmitotic maturation and the correct elaboration of their dendritic arbours [144].

A recent study showed that Olig3 and Olig2 play an essential function in safeguarding the specification of Purkinje cells by curtailing a transcriptional programme characteristic of inhibitory interneurons [106]. In the ventricular zone, *Olig3* and *Olig2* are largely co-expressed with Ptf1a+ progenitor cells between E11.5 and E13.5 (Fig. 3C), after which the number of Olig3+ and Olig2+ cells sharply decline. Histological analyses in developing mice show that *Olig2* expression is restricted to Ptf1a+ progenitor cells, whereas *Olig3* expression is initiated in proliferative Ptf1a+ cells and is transiently retained in early born Purkinje cells (Fig. 3C) [106,145–147]. Newborn Purkinje cells and inhibitory interneurons differentially express the transcription factors *Foxp2* and *Pax2*, respectively (Fig. 5A,B) [106,148]. The ablation of *Olig3* and *Olig2* results in the supernumerary development of inhibitory interneurons at the expense of Purkinje cells (Fig. 5C) [106,145,146]. Interestingly, it has been recently demonstrated that the loss of these two bHLH factors de-represses *Pax2* in newborn Purkinje cells, which erroneously co-express *Foxp2* and *Pax2* (schematically displayed in Fig. 5D) [106]. Furthermore, these mis-specified Foxp2+/Pax2+ Purkinje cells undergo a fate-shift and become inhibitory interneurons, a process that is cell-autonomously instructed by Pax2. Thus, Olig3 and Olig2 secure the correct development of Purkinje cells by preventing them from acquiring an inhibitory interneuron identity. Indeed, Lowenstein et al. show that the ectopic expression of *Olig3* is sufficient to suppress *Pax2* expression in the ventricular zone and the development of inhibitory interneurons, a phenotype that also seems to be recapitulated by the ectopic expression of *Olig2* [106,146].

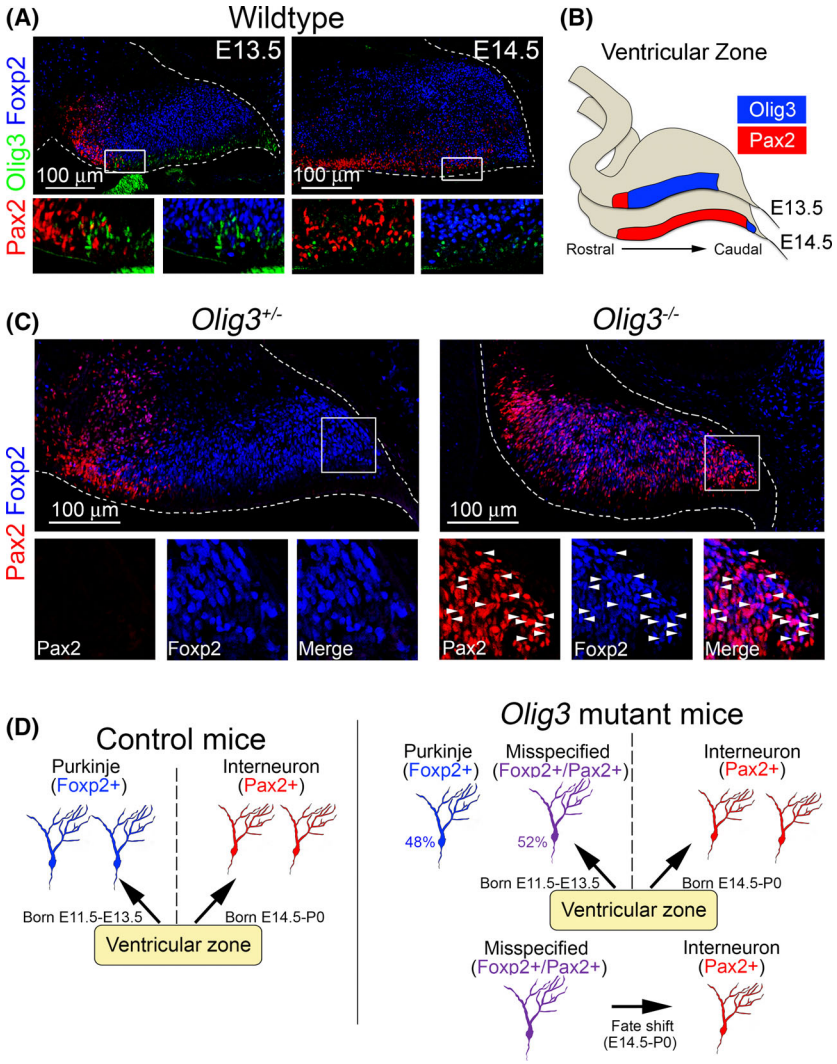

Fig. 5. Purkinje cell development. (A) Immunofluorescence characterization of Foxp2+ (blue) Purkinje cells, Pax2+ (red) inhibitory interneurons and Olig3+ (green) progenitor cells in wild-type mice at the indicated stages. Boxed areas are magnified underneath the main photographs. (B) Schema illustrating the development of Pax2+ inhibitory interneurons. At E13.5, inhibitory interneurons develop in a rostral domain of the ventricular zone that lacks Olig3 expression. At E14.5, Pax2+ cells span most of the ventricular zone as Olig3 expression becomes extinguished. (C) Sagittal cerebellar sections taken from *Olig3*$^{GFP/+}$ (control) and *Olig3*$^{GFP/GFP}$ (mutant) mice at E13.5. The sections were stained against Pax2 (red) and Foxp2 (blue). The boxed areas are displayed to the bottom of the main photographs illustrating individual and merged fluorescent signals. Note that numerous Foxp2+ cells co-express Pax2 (arrowheads) in *Olig3* mutant mice. (D) Schema illustrating the above findings. In control mice, the ventricular zone generates two sets of GABAergic neurons: Foxp2+ Purkinje cells (E11.5-E13.5) and Pax2+ inhibitory interneurons (E14.5- P0). In *Olig3* mutant mice, about half of the Foxp2+ cells are misspecified and co-expressed Pax2. These cells subsequently undergo a fate shift and transform into inhibitory interneurons. This figure is adapted from Lowenstein et al., 2021 (ref [106]) that was published under the terms of the Creative Commons Attribution License, which permits unrestricted use and redistribution provided that the original author and source are credited (https://creativecommons.org/licenses/by/4.0/).

To date, little is known about the molecular mechanisms that allow the transition from Purkinje cell generation to the production of cerebellar inhibitory interneurons, although the expression of the homeodomain transcription factor *Gsx1* coincides with the receding of *Olig3*/*Olig2* in ventricular zone progenitors and the genesis of inhibitory interneurons [106,146]. Indeed, forced expression of *Gsx1* is sufficient to induce a Pax2+ inhibitory neuron fate at the expense of Purkinje cells in mice [146]. Thus, the differential expression of three transcription factors drives the generation of the two major neuronal cell types of ventricular zone, namely, *Olig3*/*Olig2* (for Purkinje cells) and *Gsx1* (for inhibitory interneurons).

A question that remains to be answered is whether the ventricular zone contains two distinct progenitor cells that exclusively generate Purkinje cells or inhibitory interneurons, or whether the same progenitor cells can transit from generating Purkinje cells to interneurons. Examples for both possibilities are abundant during the nervous system development. For instance, cortical interneurons are generated from various distinct pools of progenitor cells [149,150]. In contrast, brainstem serotonergic neurons develop from progenitor cells that first produce visceral (and branchial) motor neurons. The temporal shift between the generation of branchio/visceromotor neurons to serotonergic neurons correlates with the receding of *Phox2b* expression in those progenitor cells [151–153]. In 2014, Seto et al. postulated a 'temporal identity transition' model, based on detailed short-term lineage-tracing experiments, in which Olig2+ Purkinje cell progenitors transition into inhibitory interneuron progenitors [146]. From this model, one would expect that inhibitory interneurons would have a history of *Olig2* expression. However, the current evidence appears to challenge this model, as long-term lineage-tracing experiments using $Olig2^{cre}$ and $Olig3^{creERT2}$ mice showed that Pax2+ inhibitory interneurons rarely have a history of *Olig2* or *Olig3* expression, even though both factors are abundantly co-expressed in Ptf1a+ progenitor cells [106,145]. One possible explanation for this discrepancy is that *Olig2*/*Olig3* might become expressed in committed progenitors that will leave the cell cycle and no longer proliferate, thereby preventing the labelling of inhibitory interneurons with $Olig2^{cre}$ and $Olig3^{creERT2}$ lineage-tracing. However, there are two pieces of evidence that argue against this possibility: (a) *Olig3* is expressed in most ventricular zone proliferative cells during the development of Purkinje cells and (b) the ablation of *Olig3*/*Olig2* does not result in the specification of inhibitory interneurons but rather in the misspecification of Purkinje cells, which are later transformed into inhibitory interneurons by the action of Pax2 [106]. Therefore, we hypothesize that two progenitor cell types might exist in the ventricular zone, one active during the generation of Purkinje cells and a second one quiescent in early development, which later generates inhibitory interneurons as Purkinje cell progenitors become extinguished.

Cerebellar inhibitory interneurons have been classically defined according to their morphology and location as Lugaro and Golgi cells (in the granular layer) and stellate and basket cells (in the molecular layer). Birth-dating experiments indicate that Lugaro and Golgi cells develop first (E14.5-E16.5), whereas stellate and basket cells develop later (E16.5-birth), implying that cerebellar inhibitory neurons are generated in an inside-first/outside-after manner [82,154]. One should note that inhibitory interneurons radially migrate from the ventricular zone upon their specification and accumulate underneath the developing Purkinje cell layer before settling into their final position in the cerebellar cortex in the early days of postnatal life [155–158]. To date, little is known about the molecular machinery operating during the specification of the distinct subtypes of cerebellar inhibitory interneuron. However, the development of these cells depends on the expression of *Gsx1* and *Pax2*, which is first detectable in the rostral part of the ventricular zone at E12.5 in mice and extends throughout the entire ventricular zone from E14.5 onwards [106,146]. The ablation of *Ascl1* has been reported to severely impact the development of late born inhibitory interneurons, that is stellate and basket cells, which might result from an imbalance in the proliferation of ventricular zone progenitors [142]. The ablation of *Ascl1* also compromises the generation of cerebellar glial cells, including both astrocytes and Bergman glia (mainly after birth in mice), which derive from the ventricular zone and are reduced in numbers in *Ascl1* mutant mice [142,159,160].

In conclusion, the temporal development of ventricular zone derivatives depends on the co-expression of *Ptf1a* with *Olig3*/*Olig2* (to specify early born Purkinje cells) and *Ptf1a* with *Gsx1* (to specify late born inhibitory interneurons) (schematically summarized in Fig. 6).

Granule cell development

Granule cells represent the most abundant neuron type in the cerebellum and even in the entire nervous system [130,161]. The development of this neuronal population can be traced back to the specification of their precursor EGL cells in the rhombic lip as early as E12.5 in mice (see above). The specification of EGL cells depends on intrinsic rhombic lip and extrinsic cell mechanisms. For instance, the expression of *Lmx1a* in the roof plate and rostral rhombic lip is critical for the generation of the choroid plexus and the specification of a subset of EGL cells destined to populate the cerebellar vermis [97–99,102,162,163]. In particular, the ablation of *Lmx1a* leads to the precocious exit of EGL cells from the rhombic lip, which change their location from the posterior to the anterior vermis [67,68,70,98,99]. Strikingly, the genetic elimination of the roof plate results, among several pronounced hindbrain phenotypes, in the almost complete absence of Atoh1+ cells in the rostral rhombic lip and its consequent absence in EGL cells [67,68,70,98,99]. *Atoh1* is

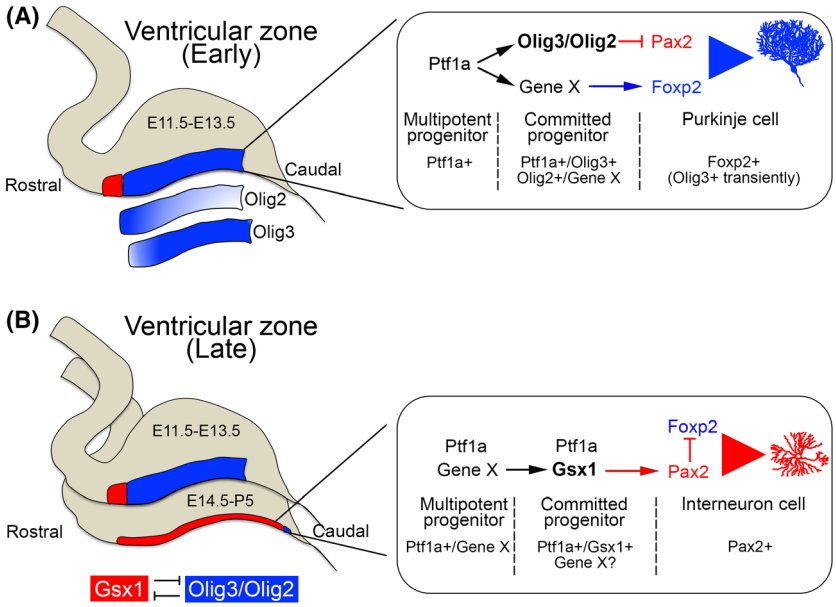

Fig. 6. Cerebellar GABAergic neuron specification. (A, B) Schematic displays illustrating early (A) and late (B) development of ventricular zone GABAergic neuronal derivatives. In early development (E11.5-E13.5), the induction of Olig3 and Olig2, in committed Ptf1a+ progenitor cells, curtails the expression of Pax2 to allow for the correct specification of Foxp2+ Purkinje cells. In late development (E14.5-first days of postnatal life), the induction of Gbx1, in committed Ptf1a+ progenitor cells, activates the expression of Pax2 to allow for the specification of Pax2+ inhibitory interneuron cells. This figure is adapted from Lowenstein et al., 2021 (ref [106]) that was published under the terms of the Creative Commons Attribution License, which permits unrestricted use and redistribution provided that the original author and source are credited (https://creativecommons.org/licenses/by/4.0/).

key in the cell-autonomous specification of EGL cells, the mutation of which leads to the complete absence of EGL cells and massive cerebellar hypoplasia [89,93,94,96,107–109].

As described above, EGL cells locate beneath the pial surface, where they form a largely homogenous multilayer of transit amplifying progenitors characterized by their expression of *Neurod1*, *Pax6*, *Insm1*, *Zic1* and *Zic3*, in addition to *Atoh1*. While the ablation of *Neurod1*, *Pax6* or *Insm1* does not alter the specification of EGL cells, the mutation of these genes does compromise their subsequent differentiation into their postmitotic granule cell derivatives [10,114–117,164,165]. EGL cells actively proliferate between E13.5 and the third week of postnatal life in mice. During this time, EGL cells undergo an enormous clonal expansion that Giacomo Consalez has estimated to be about a 3000-fold increase in their cell numbers [161]. The clonal expansion undergone by EGL cells directly depends on mitogenic signals produced by Purkinje cells and, in particular, on the diffusible morphogen and mitogen Sonic hedgehog [166–170]. As with other neurogenic niches in the nervous system, the proliferation of EGL cells is also regulated by Notch signalling [118,120]. For instance, the induction of *Notch2* via its Jagged1 ligand stimulates EGL cell proliferation, while preventing their differentiation in granule cells [118,120,171]. At the end of EGL clonal expansion, it has been estimated that each of these precursors might generate up to 250 postmitotic granule cells requiring about eight cell divisions [172]. Upon leaving the cell cycle granule, cells initiate an inward radial migration from the EGL cell layer to the granule cell layer, where they finally reside in adult life, using the apical processes of the Bergmann glia as a scaffold [173–177].

Cerebellar development at the single cell level

From the minimalistic point of view, the cerebellum consists of three distinct excitatory neuron (DCN neurons, granule and unipolar brush cells) and two inhibitory neuron (Purkinje cells and interneurons) types. Similar to other regions in the nervous system, cerebellar neuron diversity has been classically defined by their morphological, electrophysiological and chemical characteristics. The advent of scRNA-seq in cerebellar development is further expanding our knowledge on the elementary basis regulating progenitor cell progression and neuron specification. Carter et al. (2018) used

scRNA-seq and sampled almost 40 000 cells across embryonic and postnatal development to produce a transcriptional atlas of cerebellar neuron development [178]. As expected, the authors observed progenitor cells whose numbers were highly represented in the early timepoints, and their numbers decreased as they generated cerebellar neurons. More recently, Serrapolous and colleagues (2021) examined *cis*-regulatory element activity across cerebellar development using single nucleus assay for transposase accessible chromatin (snATAC-seq) and showed that despite the fact that glutamatergic and GABAergic neurons develop from the rhombic lip and ventricular zone, respectively, and express unique gene expression programmes, their progenitor cells cluster together according to their developmental stage, and not by their germinal zone [179]. This snATAC-seq study profiled 90 000 cerebellar cells across roughly the same embryonic and postnatal timepoints as the scRNA-seq atlas from Carter et al., 2018. Therefore the snATAC-seq and scRNA-seq data could be integrated and used for cell-type analyses. This revealed that many *cis*-regulatory elements are active across many early born neuron types and thus display pleiotropic activity during early cerebellar development. These findings suggest that there must be common elements, such as secreted factors or cell-cell signalling, that broadly influence cerebellar progenitor cell fate decisions in an invariant temporal specific manner. In support of this, recent work has revealed that Notch signalling between Sox2+ cerebellar progenitors at the VZ/RL boundary determines whether they give rise to glutamatergic (Atoh1+, low Notch) or GABAergic (Ptf1a+, high Notch) cells [120].

Carter at al. (2018) also showed the utility of their single cell data by elucidating the transcriptional programmes involved in glutamatergic progenitor cell fate decisions. For instance, the analysis of transcription factors expressed in five clusters that corresponded to DCN neurons and EGL cells produced a pseudotime tree with a single branch point leading towards DCN neurons or EGL cells and revealed genes that were expressed in common glutamatergic progenitors (*Atoh1*, *Barhl2* and *Insm1*), high in EGL cells (*Neurod1*, *Pax6*, *Sp5*), as well as high in DCN neurons (*Pbx3*, *Sox4*, *Neurod6*). *Sp5* also came up in an orthogonal analysis that explored the correlation network of transcription factors in glutamatergic clusters, suggesting that *Sp5* might have a central role in the differentiation and specification of EGL cells. Interestingly, *Sp5* has been shown to be downstream of the Wnt signalling pathway and to act as a repressor of genes during neural crest development, perhaps playing a similar role in preventing the premature specification of EGL cells during cerebellar development [180].

Wizeman and colleagues (2019) profiled the transcriptomes of 9,400 cells at E13.5, a dynamic time point in cerebellar development, where many different cell types are actively proliferating and differentiating. They found five transcriptomically unique subtypes of Purkinje cells (defined by their expression of *Etv1*, *Nrgn*, *En1*, *Cck* and *Foxp1*) [102]. Immunofluorescence revealed that these subtypes occupy distinct regions in the cerebellum and could also be identified at E18.5 [102]. Therefore Purkinje cell subtype identity is specified shortly after leaving the cell cycle, and this might determine their final spatial location. A more recent snRNA-seq atlas of the adult cerebellum, by Kozareva and colleagues (2021), profiled more than 600 000 cells and further expanded the number of Purkinje cell subtypes in the adult to nine [130]. It would be interesting to see how the five subtypes of PC discovered by Wizeman et al. (2019) at E13.5 relate to the clusters found in the adult by Kozareva et al., 2021.

Kozareva et al. (2021) also examined the development of molecular layer inhibitory interneurons, which have been historically separated into stellate and basket cells based on their morphology [82,130,154]. This distinction is a little unclear however as many of these interneurons display mixed morphologies, leading to the conclusion that these cells may represent a morphological contiuum. Surprisingly, their adult snRNA-seq data revealed two classes of molecular layer interneurons, which they termed as class I (MLI1 and *Sorcs3*+) and class II (MLI2 and *Nxph1*+). In an effort to examine the development of these interneurons, the authors expanded their analysis to 5500 GABAergic progenitors across four stages of development and could distinguish between the distinct developmental trajectories of class I and class II interneurons. Although these cells develop from common progenitors, class II differentially expressed immediate early genes such as *Fos* during their development, which the authors concluded might indicate a higher cellular activity during their specification. Both classes of interneurons differ in their electrophysiological properties, and many class I, but not class II, are coupled to one another via gap junctions, demonstrating that the transcriptomic differences between these two classes of interneurons are also functionally relevant.

scRNA-seq has also led to inroads in understanding medulloblastoma, a deadly paediatric brain cancer that originates in the cerebellum. Although gene expression and DNA methylation determined that medulloblastoma is comprised of four molecularly distinct

subtypes, namely sonic hedgehog, Wnt, Group 3, and Group 4, the exact contribution of different cerebellar lineages to these subtypes was previously unclear [181]. Whereas sonic hedgehog and Wnt types of medulloblastoma emerge from deficits affecting these signalling pathways, the aetiology of Group 3 and Group 4 medulloblastoma remains to be elucidated [182]. The identification of the exact cellular origin of these tumour subtypes could lead to novel treatment strategies that target the specific cell types or developmental pathways involved. In 2019, two independent groups of scientists used scRNA-seq to determine whether the four subtypes of human medulloblastoma derive from common or distinct cerebellar populations [183,184]. In doing so, both groups uncovered the developmental origin of each of the four subtypes. For instance, Sonic hedgehog medulloblastoma, which accounts for 33% of all cases of this pathology, was found to derive from EGL cells. Interestingly, sonic hedgehog medulloblastoma showed transcriptomic heterogeneity that can be subdivided into three transcriptionally unique types. The first was defined by the expression of cell cycle genes such as TOP2A and CDK1, the second expressed translation initiation and elongation factors such as EIF3E and EEF1A1 as well as PTCH1 and BOC, while the last expressed genes involved in neuronal differentiation such as STMN2 and TUBB2B [183,184]. When comparing the data sets generated by these two groups with scRNA-seq data from the developing mouse cerebellum, the authors found that the second type of sonic hedgehog medulloblastoma correlates with undifferentiated unipolar brush cell and EGL cell types, while the third type of sonic hedgehog medulloblastoma correlates with differentiated granule cell types [183,184]. In an animal model that recapitulates sonic hedgehog medulloblastoma, tumour-like EGL cell types were found to have significantly increased expression of cell cycle and progenitor related gene pathways [185]. The upregulation of these pathways in transformed EGL cells led them to have significantly less propensity to differentiate, and enhanced their tumourgenicity [185].

Conclusion

Given the enormous diversity and complexity of the central nervous system, the molecular mechanisms that safeguard the development and function of its individual neuronal components has been a matter of great interest. Since the turn of the 20th century, enormous progress has revealed many of the molecular mechanisms operating in cerebellar development. To date, the basic molecular machinery required for the specification of the major cerebellar neuron populations has been identified. Yet, several pieces of the cerebellar development puzzle remain to be elucidated. For instance, the molecular determinants that distinguish the specification of the nine newly discovered types of Purkinje cells are still unclear [130]. The use of next generation sequencing techniques now allows us to study the dynamic changes in gene expression that progenitor cells undergo during their differentiation with unprecedented detail and will hopefully address similar questions soon. Future work should build on the recent cerebellar sequencing data sets to generate testable hypotheses that can shine a light on the nuances of cerebellar cell type development. A recent study in this line was published by Bayin and colleagues where they used scRNA-seq combined with elegant lineage-tracing experiments to reveal that upon injury to the neonatal cerebellum, a normally gliogenic progenitor induces expression of *Ascl1*, undergoes a glia-to-neuron transition and generates granule cells, thus restoring cerebellar development [186]. Future studies are required to discern the mechanisms underlying several cerebellar conditions that emerge during development, such as congenital cerebellar malformations, which frequently lead to a lack of coordination, a condition known as ataxia, which also includes gait abnormalities, speech deficits and disorganized eye movements.

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Conflict of interest

The authors declare that no conflict of interest exists.

Author contributions

EDL, KC and LRH-M wrote the original draft and reviewed the literature. All authors contributed to the article and approved the submitted final version.

Data availability statement

Data sharing is not applicable to this article as no new data were created or analysed in this study. The reference from which Figures 3-6 were adapted is provided in the corresponding legends.

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