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REVIEW

Development of the cerebellum: simple steps to make a 'little brain'

Thomas Butts[1,2,*], Mary J. Green[3,*] and Richard J. T. Wingate[1,‡]

ABSTRACT

The cerebellum is a pre-eminent model for the study of neurogenesis and circuit assembly. Increasing interest in the cerebellum as a participant in higher cognitive processes and as a locus for a range of disorders and diseases make this simple yet elusive structure an important model in a number of fields. In recent years, our understanding of some of the more familiar aspects of cerebellar growth, such as its territorial allocation and the origin of its various cell types, has undergone major recalibration. Furthermore, owing to its stereotyped circuitry across a range of species, insights from a variety of species have contributed to an increasingly rich picture of how this system develops. Here, we review these recent advances and explore three distinct aspects of cerebellar development – allocation of the cerebellar anlage, the significance of transit amplification and the generation of neuronal diversity – each defined by distinct regulatory mechanisms and each with special significance for health and disease.

KEY WORDS: Granule cell, Atoh1, Autistic spectrum disorder, Medulloblastoma, Ptf1a, Purkinje cell

Introduction

The cerebellum ('little brain') resides at the anterior end of the hindbrain and is classically defined by its role in sensory-motor processing (Buckner, 2013). In amniotes, it represents one of the most architecturally elaborate regions of the central nervous system (CNS), and in humans it contains over half of the mature neurons in the adult brain (Butts et al., 2012). This morphological complexity belies histological simplicity: the cerebellar cortex is composed of a very basic structure comprising a monolayer of inhibitory Purkinje cells (see Glossary, Box 1) sandwiched between a dense layer of excitatory granule cells (see Glossary, Box 1) and a sub-pial molecular layer of granule cell axons and Purkinje cell dendritic trees (Fig. 1). Granule cells receive inputs from outside the cerebellum and project to the Purkinje cells, the majority of which then project to a variety of cerebellar nuclei (see Glossary, Box 1) in the white matter. A less well-defined complement of locally interacting inhibitory interneuron cell types and glutamatergic unipolar brush cells (see Glossary, Box 1) complete the circuit, which famously promised to be the first of any vertebrate neural network to be fully comprehended (Eccles et al., 1967).

At around the same time as Eccles, Ito and Szentágothai were publishing their famous treatise on the cerebellum as a neural machine (Eccles et al., 1967), the variation in cerebellar structure across vertebrates emerged (Fig. 2), thus highlighting the cerebellum as an important model for brain evolution (Nieuwenhuys, 1967). With its various morphogenic manifestations clearly representing variations on a simple theme, the cerebellum provided the perfect template for addressing adaptive developmental processes. However, the failure of comparative anatomy to deliver on a mechanism resulted in insights of the late 1960s languishing unattended in intervening years.

Historically, studies of the cerebellum focussed on its description through fate mapping, its induction via FGF signalling or its role as a locus for developmental cancer. However, in recent years, each of these perspectives has been subject to a more or less severe reworking, and this revision has generated important insights into the organisation of neurogenesis, the cell lineages, temporal patterning and differentiation in the cerebellum. Collectively, this scrutiny has propelled the cerebellum into a pre-eminent model for neural development, an understanding of which impacts on a range

Box 1. Glossary

Actinopterygian fish Ray-finned fish. One of the two branches of extant osteichthyeans (bony vertebrates) that comprises all of the extant fish with the exception of the coelacanth and lungfish. The latter two, together with tetrapods, make up the other branch of bony vertebrates: the lobe-finned fish (sarcopterygians).

Bergmann glia. A characteristic glial population of the cerebellum. In development, they function as scaffolds for the radial migration of granule cell precursors from the EGL to the IGL.

Cerebellar nuclei. Clusters of glutamatergic and GABAergic neurons located in the cerebellar white matter that are the synaptic targets of the majority of Purkinje cells. Projection neurons within nuclei account for the output of the cerebellum. Cerebellar nuclei are often termed 'deep' although the designation is superfluous.

External germinal layer (EGL). A transient zone of granule cell precursors that is formed from cells that migrate tangentially from the rhombic lip to cover the pial surface of the developing cerebellum during development. Subsequently, cells of the EGL migrate radially to their final position as mature granule cells within the internal granule layer.

External 'granule' cell layer. A common, if less precise, substitution for external germinal layer. It adequately describes a transient superficial layer of an embryonic cerebellum that is either non-proliferative (amphibian) or proliferative (birds and mammals) but does not discriminate between the two.

Granule cells. Glutamatergic excitatory neurons in the internal granule layer that receive excitatory inputs from mossy fibres, the majority of which originate in the pons, medulla and spinal cord. They receive local inhibitory inputs from Golgi neurons. Granule cells extend T-shaped axons into the molecular layer where they synapse with Purkinje cell dendrites.

Medulloblastoma. A developmental tumour that originates either from cells within the cerebellum or the dorsal hindbrain.

Purkinje cells. GABAergic inhibitory neurons in the cerebellar cortex that receive excitatory inputs from granule cell parallel fibres and inhibitory input from climbing fibres of the inferior olive. The majority of Purkinje cell axons project to the deep cerebellar nuclei, while a subset directly innervates vestibular targets in the hindbrain.

Unipolar brush cells. Glutamatergic interneurons that are found in the internal granule layer in areas associated with the vestibular system. They receive inputs from the vestibular system nuclei in the hindbrain and project locally to granule cells.

[1]MRC Centre for Developmental Neurobiology, King's College London, London SE1 1UL, UK. [2]School of Biological and Chemical Sciences, Queen Mary, University of London, London E1 4NS, UK. [3]National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.
*These authors contributed equally to this work

‡Author for correspondence (richard.wingate@kcl.ac.uk)

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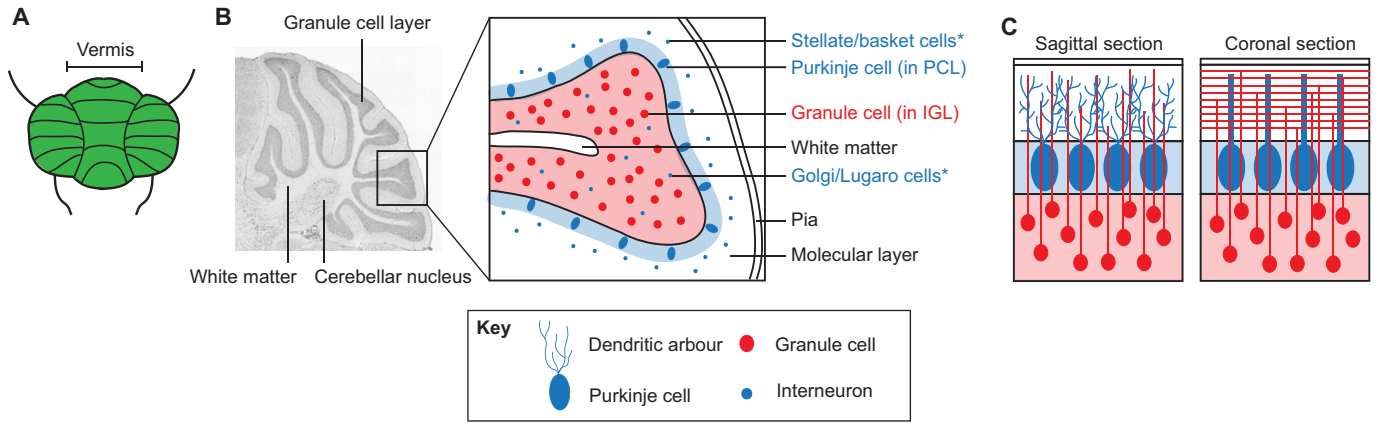

Fig. 1. Structure of the cerebellum. (A) Viewed superficially, the cerebellar is divided into transverse folia. The mammalian cerebellum (green) is characterised by a medial expansion of the hemispheres into a vermis. (B) In sagittal section, each folia comprises distinct cellular layers with white matter beneath. Cerebellar nuclei lie within the white matter. Layering reflects the distribution of different cell types: Purkinje cell layer (blue), internal granule cell layer (red) and a molecular layer (not coloured) in which Purkinje cell dendrites and granule cell axons interact. Each layer also contains characteristic GABAergic interneuron subtypes (*). Of these, only the stellate neuron appears to be present in all vertebrates, whereas others have a variable distribution: Lugaro (mammals only), basket (birds and mammals), Golgi (birds, reptiles and mammals) (Llinás and Hillman, 1969). Glutamatergic interneurons (unipolar brush cells) have also been found in the IGL in both birds and mammals (Takacs et al., 1999). (C) Schematic magnified views (sagittal and coronal sections) of the molecular layer of the cerebellum. Granule cell axons form parallel fibres arranged orthogonally to Purkinje cell dendritic arbours.

of congenital and acquired disorders. The increasing recognition of the diversity of cerebellar-related syndromes reflects a growing understanding of the repertoire of brain regions influenced by cerebellar activity, as revealed by novel mapping techniques and implied from clinical studies. Most recently (Courchesne et al., 1988; Brito et al., 2009; Schmahmann, 2010; Buckner, 2013), the cerebellar circuit has achieved a new significance in the context of autistic spectrum disorder (ASD). Moreover, the recent explosion of genetic developmental techniques means that the cerebellum can perhaps fulfil its potential as a model in comparative approaches in biology.

In this Review, we outline the advances made over this decade in understanding cerebellum development and discuss their significance for clinical science. Using insights gained from studies of sharks, paddlefish, zebrafish, frogs, chicks and mice, we focus on three distinct aspects of cerebellar development that represent autonomous phases of growth: the allocation of the cerebellar anlage, the significance of transit amplification and the generation of neuronal diversity.

An overview of cerebellar development

Although it is easiest to consider how developmental phases fit together in the mammal, it is important to recognise that, beyond the stereotyped neuronal Purkinje-granule cell circuit, evolutionary variability in cerebellum form reflects variability in how these phases are deployed in the embryo. Thus, the territory that will generate the cerebellum – its 'anlage' – is allocated during the early embryonic segmental phase of hindbrain development [in mouse, at approximately embryonic day (E) 8.5] close to the boundary (the 'isthmus') between the hindbrain and the midbrain. However, as we will describe, regulation of patterning in this earliest phase seems particularly important for the development of the uniquely mammalian midline expanded region of the cerebellum known as the 'vermis' (Fig. 1A).

Lagging behind the establishment of rhombomere boundaries (Simon et al., 1995), specific cell types are allocated along the dorsoventral axis. For glutamatergic cells of the cerebellum, this is a remarkably prolonged and, importantly, a dynamic process that takes place at the most dorsal interface between neural and

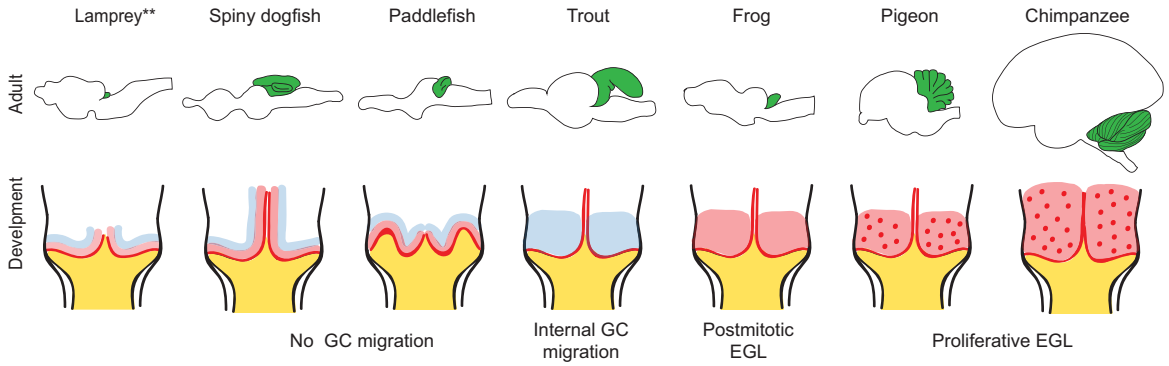

Fig. 2. Variations in cerebellar morphology. Variation in the morphology and of the adult cerebellum (green) across vertebrates is reflected in developmental adaptations of the granule cell precursor pool (red). Cerebellar expansion in basal fish corresponds to linear extensions of the rhombic lip axially (spiny dogfish) or medially (paddlefish). In other clades, granule cells (pink shaded area) are distributed in an internal layer that is co-extensive with the overlying Purkinje cells (blue). To achieve this, granule cells migrate internal to (teleosts and tadpoles) or external to (metamorphic amphibians, birds and mammals) and then through the Purkinje cell layer. Only in birds and mammals do granule cell precursors themselves migrate in substantial numbers to form a transient external germinal layer. **A theoretical model of the as yet uncharacterised embryonic lamprey.

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non-neural 'roof plate' tissue: the rhombic lip (in mouse, at E10.5-E18.5). This phase generates the basic dichotomy between GABAergic and glutamatergic cell types that underlies the conserved Purkinje-granule cell circuit, but, as we will see, it is also responsible for the diversity of cerebellum output connectivity across species.

Cell type allocation precedes a third, distinct temporal phase of development that extends into early prenatal life (postnatal day 21 in mouse and up to 2 years in humans). Here, the principal derivative of the rhombic lip, the granule cell precursor, accumulates over the surface of the cerebellum and undergoes further rounds of symmetric divisions in a process of transit amplification that exponentially expands its numbers. Growing evidence suggests that this most investigated phase of cerebellum development is substantially reduced or absent in aquatic vertebrates (Fig. 2). Because the final form of the mammalian cerebellum is so much a product of the first and third phases of development, we will consider these first before looking at the less well-understood process of cell type allocation.

Defining the cerebellar anlage: molecular boundaries and the role of Fgf8

A fundamental determinant of cerebellar morphology is the allocation of a territory in which its component cell types are specified. Despite the distinct structure and clear boundaries of the cerebellum, this simple problem has proved more enduring than might have been anticipated. Similarly, the association of cerebellar induction with the diffusible morphogen fibroblast growth factor 8 (Fgf8) has acquired a more nuanced perspective. It seems likely that FGF signalling has far-reaching evolutionary and developmental significance for other aspects of brain development, such as the allocation of isthmic territory and the origins of the mammalian vermis, tying embryonic events at the early stages of axial specification to surprisingly profound clinical consequences for higher cognitive function (Box 2).

The cerebellar anlage sits between Hox and Otx domains

The anlage of the cerebellum is a product of the mechanisms of segmentation that establish iterated rhombomeric subdivisions within the early hindbrain just after neural tube closure. The establishment and maintenance of the boundaries defining the territory of the cerebellum has been a subject of several recent studies. These have built our current understanding that all of the cells of the cerebellum arise from dorsal rhombomere 1 (r1), a region that is definitively characterised by an absence of the expression of Otx and Hox genes (Fig. 3). Early studies using quail-chick grafting to map boundaries of the neuromeres of the brain concluded that the majority of the cerebellum arises from the metencephalic (rostral) hindbrain, but that as much as one-third of cerebellar granule cells originate from the mesencephalon, which is rostral to the midbrain-hindbrain constriction (Hallonet et al., 1990). Later, this idea was overturned by instead mapping the molecular boundary between the midbrain and hindbrain as the caudal extent of *Otx2* expression (Millet et al., 1996), showing that all cerebellar cells are born from *Otx2*-negative tissue and also demonstrating a surprising degree of anisotropic growth proximal to the midbrain hindbrain-boundary (MHB). The caudal boundary of cerebellar territory has also been mapped by chimeric grafting to the r1/2 boundary, as marked by *Hoxa2* expression (Wingate and Hatten, 1999).

The discovery that molecular, rather than morphological, boundaries are crucial in determining cerebellar territory was soon followed by studies looking at the function of these genes in determining the fate of their respective territories: *Otx2* at the rostral boundary, *Hoxa2* at the caudal boundary and *Gbx2* expressed in r1, abutting *Otx2* and genes expressed at the MHB organiser. *Otx2* is required from an early stage to establish forebrain and midbrain territories, and its absence causes a rostral expansion of the cerebellar *Gbx2*-positive territory at the expense of midbrain tissue (Acampora et al., 1997). Conversely, ectopic expression of *Otx2* in the rostral hindbrain transforms this region into a *Gbx2*-negative midbrain identity with a caudal shift in the position of the MHB (Broccoli et al., 1999; Katahira et al., 2000). Most recently, it has been shown that the conditional deletion of *Otx2* throughout the dorsal midbrain, leaving the MHB intact, is sufficient to disrupt the differentiation of midbrain cell types and induce a program of cerebellar development in the dorsalmost region of the midbrain (Di Giovannantonio et al., 2014). Thus, it is an absolute requirement that *Otx2* is absent for cerebellar differentiation to begin.

In a similar manner to *Otx2*, *Hoxa2* expression is crucial for determining the caudal limits of cerebellar differentiation. Loss of *Hoxa2* causes caudal expansion of the cerebellum (Gavalas et al., 1997), and ectopic *Hoxa2* expression in r1 suppresses the specification of cerebellar neurons (Eddison et al., 2004). By contrast, *Gbx2* expression in r1 is required for the formation of the cerebellum, which is replaced by an expanded midbrain in *Gbx2* mutants (Wassarman et al., 1997). However, rather than playing a direct role in cerebellar differentiation, *Gbx2* function appears to be limited to the inhibition of *Otx2*. This is conclusively demonstrated in zebrafish by the rescue of *Gbx2*-null mutations by reduction of *Otx2* expression (Foucher et al., 2006; Su et al., 2014). In experiments where *Gbx2* is overexpressed in the midbrain territory, cerebellar tissue can be induced (Millet et al., 1996; Katahira et al., 2000), but this is always coupled with downregulation of *Otx2* expression in the region. Therefore, it is likely that cerebellar differentiation occurs due to the repressive actions of *Gbx2* on *Otx2*, rather than via a specific inductive role of *Gbx2*.

Together, these studies demonstrate a key requirement for the absence of *Otx2* and *Hoxa2* expression in r1 to generate a cerebellum. However, there is also a large body of work looking at how the precise positions of these boundaries are set and maintained as lineage restriction boundaries. For example, it has

Box 2. Early cerebellar patterning defects and cognitive impairment

The past 20 years have seen an increasing awareness of the role of the cerebellum in non-motor functions (Schmahmann, 2010). These functions are reflected in the higher cognitive function defects that accompany motor dysfunction following cerebellar damage (Schmahmann and Sherman, 1998). Pre-term damage to the developing cerebellum also predicates long-term cognitive deficits (Limperopoulos et al., 2007). Furthermore, congenital deficits, in particular vermal agenesis, lead to later communicative and affective relational disorders (Tavano et al., 2007). Accordingly, a study of structural brain abnormalities in mouse models of autistic spectrum disorder (ASD) revealed cerebellar-specific disruptions (Steadman et al., 2013). Although this variety of sources suggests that early patterning defects might generate significant cognitive impairment, the most compelling evidence in support of this hypothesis is a recent analysis of a mouse model of human CHARGE syndrome. CHARGE syndrome is reflected in a cluster of congenital abnormalities including ASD-like behavioural problems in humans. In mice, mutation in the chromatin modifier Chd7 leads to a vermal hypoplasia that can be directly linked to changes in Otx2 repression at the midbrain-rhombomere 1 boundary (Yu et al., 2013). This raises the possibility that other unrecognised early patterning defects may underlie a range of human cognitive deficit syndromes (Haldipur and Millen, 2013).

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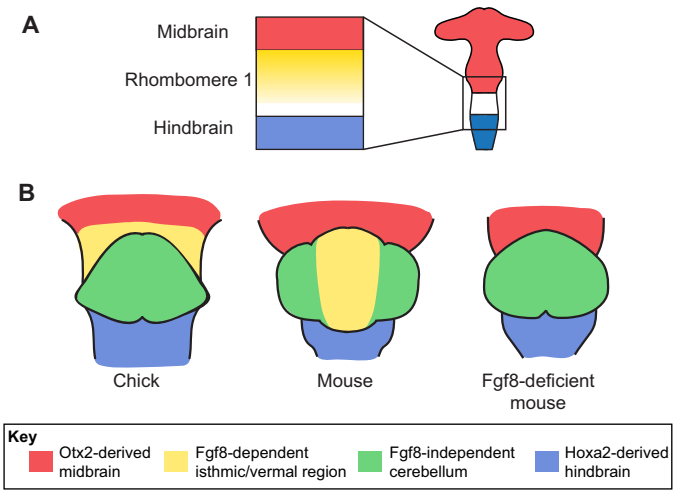

Fig. 3. FGF signalling regulates territorial allocation and anisotropic cerebellar growth from rhombomere 1. (A) In early embryonic development, the boundaries of rhombomere 1, from which the cerebellum derives, is defined by the exclusion of Otx (red) and Hox (blue) genes. FGF signalling (yellow) is established at the anterior end of rhombomere 1. (B) Colour coding indicates the contribution of territorial patterning mechanisms to regions of the adult cerebellum in birds and mammals. It seems likely that differences in their organisation reflect changes in the influence of isthmic FGF signalling on the initial expansion of the anlage. The induction of both the mammalian vermis (a medial expansion that is absent in other vertebrates) and isthmic territory, which lies just rostral to the cerebellum, is dependent on FGF (yellow). This suggests that the evolution of the mammalian vermis occurred at the expense of a more-extensive isthmic territory. Cerebellar differentiation (green) is inhibited by isthmic signalling, suggesting that FGF expands the precursor pools but is not directly involved in cerebellar specification.

been shown that distinct pathways govern the morphological and molecular features of the MHB downstream of the common transcription factor grainyhead-like 2 (Grhl2), with engrailed 2 acting downstream of Grhl2 to promote cell survival and formation of the molecular boundary (Dworkin et al., 2012). In addition to the co-repressive actions of *Otx2* and *Gbx2* at the boundary, *Fgf8*, *Gbx2* and Notch signalling (Sunmonu et al., 2011; Tossell et al., 2011) promote cell sorting and, hence, lineage restriction at the boundary. The signalling molecule Fgf8 also has a key role in establishing and maintaining the cerebellar boundary.

FGF signalling: an inducer or repressor of cerebellar development?

Fgf8 is the major signalling molecule in the MHB, and it is expressed within the *Gbx2*-positive domain and abutting *Otx2* expression (Hidalgo-Sanchez et al., 1999). Previously, it was considered that FGF signalling from this boundary induced cerebellar development, due to the ability of ectopic Fgf8 in the midbrain to induce a secondary cerebellum (Crossley et al., 1996; Liu et al., 1999; Martinez et al., 1999; Sato et al., 2001). Fgf8 is also essential for the survival of the entire midbrain-hindbrain region and is required in a dose-dependent manner for the development of the vermis (Meyers et al., 1998; Chi et al., 2003; Basson et al., 2008). However, much like *Gbx2*, FGF signalling at the MHB appears to act primarily by inhibiting *Otx2* in the r1 territory. Where reduction of *Fgf8* causes loss of the vermis, an expansion of *Otx2* expression is also seen in dorsal r1 (Sato et al., 2004; Sato and Joyner, 2009). Correspondingly, where ectopic Fgf8 induces cerebellar tissue in the midbrain territory, a downregulation of *Otx2* expression always accompanies this switch of cell fate (Liu et al., 1999; Martinez et al., 1999; Sato and Joyner, 2009). Furthermore, reduction of *Otx2* expression is sufficient to rescue the loss of the cerebellum in zebrafish *fgf8* mutants (Foucher et al., 2006), demonstrating that FGF signalling is not required to directly induce cerebellar differentiation and instead acts to maintain r1 as an *Otx2*-negative domain.

However, FGF signalling does appear to have a role beyond maintaining the caudal limit of *Otx2* expression. Blockade of FGF signalling in r1, leaving MHB signalling intact, appears to affect elongation of the rhombic lip and r1, suggesting that FGF signalling mediates growth of the territory (Green et al., 2014). Given the anisotropic nature of growth at the isthmus (Millet et al., 1996) and the rostral origin of the cerebellar vermis in r1 (Sgaier et al., 2005), it is possible that the vermal dysplasia observed in *Fgf8* hypomorphic mice may be attributed to a reduction in Fgf8-mediated growth (Fig. 3), in addition to the loss of cerebellar territory through expansion of the roof plate (Basson et al., 2008) and the *Otx2* domain (Sato and Joyner, 2009). Furthermore, in sprout 2 (*Spry2*) mutants, in which negative feedback of FGF signalling is reduced, the cerebellar vermis is expanded (Yu et al., 2011), suggesting an increase in rostral r1 growth.

Corresponding to this role as a proliferative node, the isthmic region of r1 is evolutionarily diverse. In actinopterygian fish (see Glossary, Box 1), it is the origin of a sometimes hugely elaborate and expanded valvulus (Chaplin et al., 2010; Kaslin et al., 2013). It also spawns a range of isthmic nuclei with sensory coordinating roles across different vertebrates, which develop from a newly identified FGF-dependent domain of isthmic *Atoh1* expression (Green et al., 2014). The evolutionary emergence of the mammalian vermis appears to have been at the expense of the development of a subset of isthmic nuclei (or a valvulus) (Fig. 3). This presents a paradox, given that the scale of both the isthmic structures and the vermis are dependent on FGF signalling. The resolution of this contradiction lies in recent evidence that FGF, perhaps paradoxically, inhibits cerebellar development: while FGF signalling increases the size of the cerebellar anlage, downregulation of FGF signalling is essential for the specification of cerebellar cell types (Suzuki-Hirano et al., 2010). Furthermore, overexpression of *Fgf8* drives the specification of non-cerebellar, *Lhx9*-positive cell types in early r1 and at the isthmus in favour of later-born cerebellar cell types (Green et al., 2014). Hence, the removal of FGF signalling after a period of establishing territory boundaries and promoting growth is essential for the onset of cerebellar development.

Transit amplification and the size and foliation of the cerebellum

Although early events can significantly bias patterns of cerebellar growth, the final shape and size of the cerebellum of mammals and birds (possibly all reptiles) is the product of a remarkable example of a discrete phase of transit amplification that occurs much later in development. This proliferative episode takes a small number of *Atoh1*-positive granule cell precursors and multiplies their numbers by many fold through multiple symmetrical mitoses of single fated germinal cells. The transient appearance of this population of granule cell precursors over the surface of the cerebellum was quickly identified as a key feature of cerebellum development (Ramón y Cajal, 1894) and offered an intuitive explanation for the massive foliation of the cerebellar surface in mammals. More recently, the same logic has made the outermost layer of the cerebellum, the external germinal layer (EGL, see Glossary Box 1), an obvious candidate for medulloblastoma (see Glossary Box 1), a devastating childhood cancer (Box 3). This has exemplified how

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Box 3. Medulloblastoma and the EGL

Medulloblastoma is a devastating paediatric cancer of the cerebellum. In recent years, whole genome and transcriptome sequencing of clinical samples has revealed a number of molecularly distinct subtypes of medulloblastoma (Jones et al., 2012; Pugh et al., 2012; Robinson et al., 2012) that frequently involve activation of the Shh and Wnt pathways. Disruption of transit amplification remains a compelling model for the Shh subgroup of tumours, based on experimental disruption of Shh signalling (Goodrich et al., 1997), and more recent developmental studies show that commitment to the granule cell lineage is a prerequisite for tumour formation (Schuller et al., 2008; Yang et al., 2008; Li et al., 2013). Although Wnt signalling also affects cerebellar proliferation, its effects are restricted to non-granule cells (Pei et al., 2012; Selvadurai and Mason, 2012) and accordingly the Wnt-dependent subgroup of tumours, along with some Shh subgroup tumours (Grammel et al., 2012), appears to have a hindbrain origin (Gibson et al., 2010). Pathways that might supress transit amplification, such as BMP signalling (SMAD) (Aref et al., 2013), or promote differentiation (Barhl1) (Li et al., 2004) are thus associated with improved patient prognosis (Poschl et al., 2011), in contrast to those associated with regulating granule cell precursor identity (Atoh1) (Schuller et al., 2008; Yang et al., 2008) or proliferation (Foxm1) (Schuller et al., 2007; Priller et al., 2011). Recent studies have also shown that activation of the FGF (Emmenegger et al., 2013) and Wnt pathways (Anne et al., 2013) has tumour-supressing actions. This raises the possibility that other genes that antagonise granule cell proliferation during development, such as Neurod1 (Butts et al., 2014a), may also provide a potential route to therapy.

insights from development both explain and offer therapeutic avenues for disease.

Balancing proliferation and differentiation in the external germinal layer

The EGL (Fig. 4) is defined by its transience and proliferation, and by the expression of the bHLH transcription factor Atoh1 (Akazawa et al., 1995; Ben-Arie et al., 1996, 1997), which is absolutely required both for transit amplification (Flora et al., 2009) and for supressing differentiation (Klisch et al., 2011). In mouse, the EGL persists until the third week of postnatal life, and the peak of proliferation occurs around birth (Espinosa and Luo, 2008). Ramón y Cajal was able, in his first descriptions of the developing cerebellum (Ramón y Cajal, 1894), to distinguish an outer EGL populated by proliferating progenitors and an inner EGL comprising cells that have exited the cell cycle and begun the process, of differentiation and radial migration to their ultimate destination in the internal granule layer (Fig. 4).

The tempo of transit amplification within the EGL is driven by diffusible sonic hedgehog (Shh) secreted by underlying Purkinje cells (Dahmane and Ruiz-i-Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999; Lewis et al., 2004), and the importance of this pathway in a subset of medulloblastomas has been established through a variety of experimental and genomic methodologies (Box 3). Elegant studies manipulating the Shh signalling pathway appear to confirm the idea that foliation is a product of the surface expansion generated by transit amplification (Corrales et al., 2004, 2006).

Proliferation within the EGL has also been shown to be influenced by a number of extracellular matrix (ECM) components, such as β1-integrin, that are expressed both within the EGL (Blaess et al., 2004) and in cerebellar Bergmann glial cells (see Glossary, Box 1) (Frick et al., 2012). Additionally, laminins and their α6 integrin receptor subunits are confined to the outer EGL and promote granule progenitor proliferation *in vitro*, whereas vitronectin and receptor integrin subunit α5 are confined to the inner EGL (Pons et al., 2001) (Fig. 4). Likewise, in contrast to its role in the cortical ventricular zone (Bizzoca et al., 2012), the lamina-specific expression of F3/contactin in the EGL suppresses Shh-dependent proliferation and is antagonised by its binding partner Tag1 (Xenaki et al., 2011), the deletion of which leads to ectopic subpial granule cell clusters in adult mice. Correspondingly, premature misexpression of F3/contactin attenuates granule cell progenitor proliferation (Bizzoca et al., 2003). Taken together, these data highlight that the environment that granule precursors face in the EGL is created by a balance of laminar-specific ECM components, the interactions of which await detailed dissection.

The factors governing how individual progenitors navigate this environment and terminate transit amplification are less clear and yet equally important in development and disease. The lack of an internal cell division clock (Espinosa and Luo, 2008) has focussed attention on cell non-autonomous factors such Wnt and bone morphogenetic protein (BMP) pathway signals in the EGL. For example, non-canonical Wnt signalling via Wnt3 has recently been shown to be capable of decreasing proliferation independently of BMP signalling (Anne et al., 2013). Conversely, multiple BMPs are expressed in the cerebellum during EGL development and can antagonise the Shh-dependent proliferation of granule progenitors both *in vitro* and in slice cultures (Rios et al., 2004) through regulation of *Atoh1* (Zhao et al., 2008) and via *miR22* (Berenguer et al., 2013). Conditional deletions of intracellular mediators of

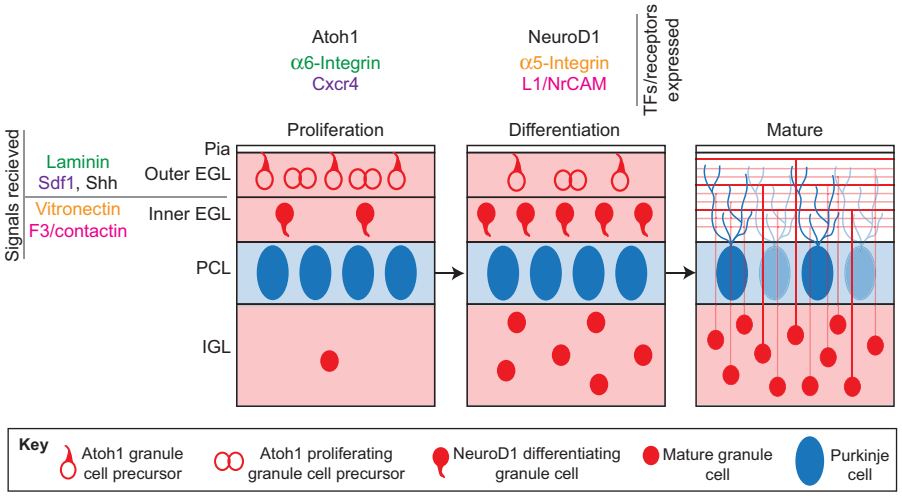

Fig. 4. Amniote granule cell progenitors proliferate in the outer EGL and begin differentiation in the inner EGL. As granule cell precursors (red) mature, they make the transition from the outer to the inner EGL, which is coincident with exit from the cell cycle. Their presence in the outer EGL is dependent upon pia-derived SDF-1 signalling via CXCR4. Although in the outer EGL, cells express the transcription factor *Atoh1*, which mediates proliferation in response to Shh secreted from Purkinje cells (blue). Subsequently, cells express *Neurod1*, downregulate *Atoh1*, and exit the cell cycle, no longer responding to Shh ligand, but instead interacting with ECM components, including vitronectin and F3/contactin, that are specific to the inner EGL. Corresponding ligands and receptors in the EGL are shown in the same colour.

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BMP signalling result in cerebellar defects (Fernandes et al., 2012; Tong and Kwan, 2013), although the interpretation of such experiments is confounded by earlier roles for BMP signalling during dorsal neural tube patterning (Alder et al., 1999; Lee and Jessell, 1999; Broom et al., 2012).

The extrinsic events that terminate proliferation may also include mechanisms that remove granule cell precursors from the sub-pial surface of the cerebellum adjacent to basal membranes. A basal lamina attachment is exhibited by all granule precursors (Hausmann and Sievers, 1985), raising the possibility that, analogous to cortical intermediate precursors in the subventricular zone (Fietz and Huttner, 2011; Molnar, 2011; Borrell and Gotz, 2014; Florio and Huttner, 2014), contact with the outer lamina is the factor that defines transit amplifying precursors. In support of this idea, the onset of radial migration is mediated by loss of responsiveness to the chemokine Sdf1, which is secreted by the meninges that surround the neural tube (Lu et al., 2001; Zhu et al., 2002, 2004; Vilz et al., 2005). Additional recent genomic analysis of mouse cerebellum following a nervous system-specific knockout of the Sdf1 receptor Cxcr4 suggests a link between changes in responsiveness to Sdf1 and the interaction with the ECM (Huang et al., 2014). This potentially mechanistically links the mode of migration to the interactions with the ECM discussed above. However, although a large number of additional pathways have been implicated in the different tangential and radial phases of granule cell migration (Chedotal, 2010), the mechanisms that mediate the decision of individual granule cells to switch their mode of migration and exit the cell cycle remain poorly understood.

Nevertheless, the presence of separable regulatory mechanisms governing the cessation of proliferation and onset of inward radial migration is exemplified in the evolution of the EGL. In the cerebellum of the amphibian, which is the simplest tetrapod cerebellum, a sub-pial granule layer forms transiently at metamorphosis over the cerebellum but fails to proliferate (Uray et al., 1987). Here, *Atoh1*-positive cells express *Neurod1*, which in amniotes is required for (Miyata et al., 1999) and is sufficient to trigger granule cell differentiation (Butts et al., 2014a), and yet are held at the cerebellar surface. As for the intermediate precursors in the EGL of birds and mammals, this layer is a transient feature of the developing cerebellum. Inward migration of amphibian post-mitotic granule cells into the internal granule layer (IGL) is triggered by thyroid hormone and correlated with the end of metamorphosis (Gona, 1972; Hauser et al., 1986).

The status of the frog external granule – as opposed to germinal (proliferative) – cell layer (see Glossary, Box 1) raises the issue of whether the original evolutionary requirement of an EGL was for proliferation alone or reflects different developmental demands. The limited number of aquatic anamniotes and pre-metamorphic amphibians so far examined lack this transient structure (Rodriguez-Moldes et al., 2008; Kaslin et al., 2009, 2013; Chaplin et al., 2010; Butts et al., 2014a,b). However, many anamniotes develop an elaborate and sizable cerebellum. This suggests that either an EGL is present in such species or that the indefinite developmental period afforded in aquatic vertebrates may alone be sufficient to generate large numbers of cerebellar neurons. If so, an external granule layer would therefore seem to be a requirement of an adaptation to land colonisation and definitive embryogenesis (Chaplin et al., 2010). One possibility is that this facilitates homogeneous distribution of granule cells within an established laminar circuitry. This implies that, within the frog, the accumulation of granule cells at the surface of the cerebellum is a means for distributing cells evenly across the anlage prior to integration into the cerebellar cortex. In such a model, transit amplification emerges as an opportunistic expedient to generate a greater number of granule cells within this transient organisation.

Box 4. ASD and cerebellar cell types

The heterogeneous nature of autistic spectrum disorder (ASD) is reflected in the range of its different, potential developmental causes. Perhaps surprisingly, the most consistent pathological correlates of ASD are found in the cerebellum (Courchesne, 1997). Furthermore, a recent meta-analysis suggests that a signature constellation of anatomical deficits makes cerebellar damage in ASD distinct from that in either ADHD or developmental dyslexia (Stoodley, 2014). These include localised folia hypoplasia (Courchesne et al., 1988) or the specific loss or alteration of Purkinje cells (Ritvo et al., 1986; Fatemi et al., 2002). Specific disruption to white matter in the superior cerebellar peduncle might be associated with a loss of cerebellar output to the thalamus (Brito et al., 2009). The dentate nucleus, which supplies this projection, is a crucial link in the cortico-cerebellar close loop circuits that potentially modulate higher cognitive functions in primates (Kelly and Strick, 2003; Strick et al., 2009) and humans (Kipping et al., 2013). The highly complex and enlarged dentate nucleus in humans shows a pronounced left-right asymmetry (Baizer, 2014) and, correspondingly, consistent unilateral reduction in dentate projections is inferred from a study of individuals with Asperger's (Catani et al., 2008). Finally, a recent transgenic study in which mutation of the tuberous sclerosis gene associated with human ASD was targeted specifically to Purkinje cells resulted in an ASD-like mouse phenotype (Tsai et al., 2012). Collectively, these observations suggest that, by virtue of cortico-cerebellar connectivity, selective cerebellar cell loss can mimic the effects of what are more readily perceived as 'cortical' syndromes (Schmahmann and Pandya, 2008).

Differentiation of progenitor zones and the generation of cellular diversity

Although the territorial allocation of the cerebellum and the expansion of granule cell numbers that shapes cerebellar morphogenesis have received a wealth of experimental scrutiny, the factors that generate cell diversity in the cerebellum have received relatively little attention. This is despite a literature that hints at important evolutionary changes in the diversity of neuronal subtypes (Llinás and Hillman, 1969; Nieuwenhuys et al., 1998) and points to a changing functional role for the cerebellum as new networks of connections emerged in amniotes. Most recently, the importance of cerebellar connectivity as a potential locus of ASD (Box 4) emphasises the need for a clear understanding of cellular specification mechanisms within cerebellar precursor pools.

Blurred lines: GABAergic and glutamatergic progenitor domains are not lineage-restriction compartments

In the same way as the definition of the territorial boundaries of the cerebellum was transformed by genetic insights, our understanding of the origins of different neuronal subtypes within the cerebellar anlage has been transformed in recent years. A key clarifying concept was identification of the origins of granule cell precursors at the rhombic lip, a thin strip of neuroepithelium that borders the non-neuronal roof plate of the fourth ventricle (Alder et al., 1996; Wingate, 2001). Although it spans the entire rhombencephalon, contributing to a variety of distinct auditory, proprioceptive and interoceptive hindbrain circuits (Rodriguez and Dymecki, 2000; Landsberg et al., 2005; Maricich et al., 2009; Rose et al., 2009), the rhombic lip of the cerebellar anlage (rhombomere 1) is the exclusive source of granule cell precursors that then migrate tangentially to form the EGL (Wingate and Hatten, 1999). The cells in the rhombic lip that contribute to the EGL already express *Atoh1*, which is induced by TGFβ signals secreted from the neighbouring

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roof plate (Alder et al., 1999; Fernandes et al., 2012; Tong and Kwan, 2013). Although this might suggest that the rhombic lip is a dorsally allocated progenitor pool, it is perhaps more appropriate to consider it as a zone of dynamic induction at the edge of the ventricular zone. The production of *Atoh1*-positive cells depends both on local Delta-Notch signalling and direct contact with the roof plate (Broom et al., 2012). Furthermore, once *Atoh1* is switched on, cells rapidly migrate away from the rhombic lip (Machold and Fishell, 2005).

Consistent with this dynamic definition of the rhombic lip as an inductive interface, the lineage boundaries between the ventricular zone, rhombic lip and roof plate are somewhat blurred (Fig. 5). The ventricular zone of the cerebellum is characterised by *Ptf1a* expression (Fig. 5A,B) and gives rise to GABAergic interneurons (Hoshino et al., 2005). By contrast, the roof plate comprises non-neural *Lmx1a*- (Mishima et al., 2009) and *Gdf7*-positive lineages (Currle et al., 2005) and gives rise to the choroid plexus (Fig. 5A,B). *Atoh1*-positive cells at the interface between these two zones are largely glutamatergic (Machold and Fishell, 2005; Wang et al., 2005; Rose et al., 2009). However, blurring is seen when genetic labelling with *Lmx1a*- and *Gdf7*-driven Cre lines, which might be expected to be confined to the roof plate and its choroid plexus derivative, is also found unexpectedly in both glutamatergic and GABAergic descendants (Chizhikov et al., 2010; Cheng et al., 2012). This is significant in that it could suggest that lineages are not restricted. Furthermore, this blurring is increased on deletion of *Lmx1a* (Chizhikov et al., 2010), whereas genetic deletion of either *Ptf1a* or *Atoh1* leads to increased mixing of lineages (Fig. 5C-E) (Wang et al., 2005; Pascual et al., 2007; Millen et al., 2014) due to the mutually repressive functions of these genes (Yamada et al., 2014). Loss of *Ptf1a* also leads to mixing between dorsal and ventral (non-cerebellar) ventricular derivatives (Millen et al., 2014). These results suggest a dynamic segregation of lineages that is dependent on their genetically determined post-mitotic identity.

As in the EGL, the proliferation of both roof-plate and ventricular-derived cells is, at late stages, sensitive to Shh signalling. In the roof plate, endogenous Shh production stimulates secondary proliferation from non-neural precursors adjacent to the rhombic lip (Huang et al., 2009; Nielsen and Dymecki, 2010). Shh secreted into the cerebrospinal fluid (Huang et al., 2010) acts to drive early ventricular zone proliferation whereas, later, Shh secreted from Purkinje cells also acts on a population of secondary precursors of inhibitory neurons and glia that reside in the prospective white matter (Leto et al., 2009; Fleming et al., 2013).

Timing and diversity: how cells become specified

Although Shh-dependent late-born populations represent the last stages of cell production in the cerebellum, a clear temporal order of cell production precedes this stage. This temporal pattern is superimposed onto dynamically maintained progenitor zones. Thus, in the rhombic lip, the production of granule cell precursors proceeds alongside that of a population of small unipolar brush cells (Kita et al., 2013) that also express the T-box gene *Tbr2* (Englund et al., 2006). This represents the final phase in a sequence of cell specification. Granule cell precursor production is preceded by the generation of glutamatergic cerebellar nuclei, which briefly express *Atoh1* but do not undergo transit amplification. The number of cerebellar nuclei varies between major amniote orders, with two in reptiles and between three and five divisions in mammals (Nieuwenhuys et al., 1998). These accumulate in a sequence with the most lateral being born first (Hagan and Zervas, 2012; Green and Wingate, 2014).

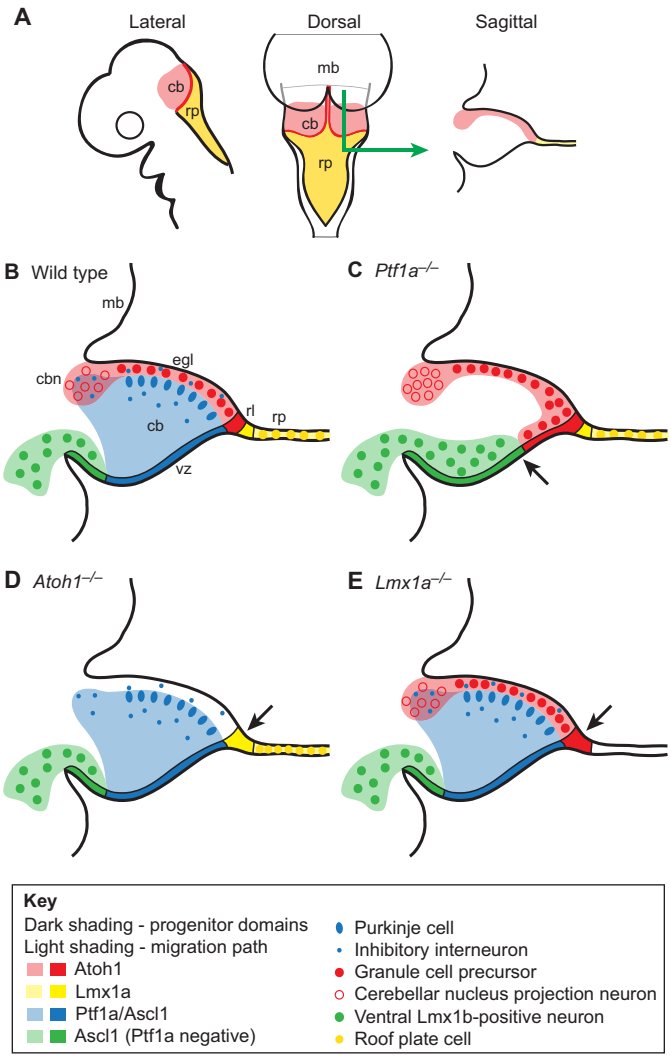

Fig. 5. Blurring the boundaries of lineages by genetic deletion of transcriptional regulators. (A) The relationship between the cerebellum (cb), midbrain (mb) and the roof plate of the IVth ventricle (rp) in a vertebrate embryo in lateral, dorsal and sagittal view. (B) A schematic sagittal section through the midbrain, cerebellum and roof plate (green line in A) showing the distinct progenitor zones at the ventricular surface (green, extra-cerebellar; blue, cerebellar; red, rhombic lip; yellow, roof plate) that contribute to different cell populations following distinct migratory paths (colour-coded shaded regions). The contributions of these progenitors zones to different cell populations are perturbed following knockdown of: (C) ventricular zone *Ptf1a* (Pascual et al., 2007; Millen et al., 2014); (D) rhombic lip *Atoh1* (Machold and Fishell, 2005; Wang et al., 2005); or (E) roof plate *Lmx1a* (Chizhikov et al., 2010). egl, external granule later; vz, cerebellar ventricular zone; rl, rhombic lip; cbn, cerebellar nuclei.

As cerebellar nuclei represent the output connection of the cerebellum, this diversity is functionally significant. For example, birds lack the most lateral of the mammalian nuclei, the *Lhx9*-positive dentate nucleus, which in mammals targets the thalamus (Arends and Zeigler, 1991; Green and Wingate, 2014). This connection allows the cerebellum to participate in regulating cortical functions and its absence in birds marks a major difference in brain organisation.

Cerebellar nucleus neurons are the first cerebellar cells to be generated, but are not the earliest *Atoh1* cells to be generated in rhombomere 1. At pre-cerebellar stages, the rhombic lip is patterned by FGF signalling from the isthmus and generates *Lhx9*-positive neurons

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that migrate into ventral and isthmic r1 (Machold and Fishell, 2005; Wang et al., 2005; Green et al., 2014), contributing cells to multiple nuclei that form part of a wider hindbrain network of nuclei controlling proprioception, interoception and arousal (Rose et al., 2009).

The switch from the production of cerebellar neurons to granule cells at E12.5 in mouse (Machold and Fishell, 2005; Wang et al., 2005) is paralleled by a switch in the production of GABAergic neurons in the ventricular zone from Purkinje cells to other types of interneurons and glia (Sudarov et al., 2011). This correlates with the changing patterns of *Olig2* and *Gsx1* expression between E12.5 and E14.5. The expression of *Gsx1*, which marks interneuron progenitors, gradually expands dorsally and into the *Olig2* lineage that, before E12.5, gives rise to only cerebellar nucleus and Purkinje cells (Seto et al., 2014). In contrast to the rhombic lip, where the outcome of a single inductive interaction changes over time, temporal patterning in the rest of the ventricular zone may reflect dynamic reorganisation of variously identified dorsoventral regions (Chizhikov et al., 2006; Zordan et al., 2008; Grimaldi et al., 2009; Mizuhara et al., 2010; Florio et al., 2012).

The correlation in the timing of fate switches and the observation that this occurs even when *Atoh1* or *Ptf1a* are misexpressed in the ventricular zone or rhombic lip, respectively (Yamada et al., 2014), suggest that a common, non-autonomous factor regulates the overall temporal development of the cerebellum and support the idea that progenitor populations share common features. Transplantation studies of both GABAergic (Leto et al., 2006, 2009) and glutamatergic rhombic lip progenitors (Wilson and Wingate, 2006) support the concept of an extrinsic cue for developmental timing. The choroid plexus, which is generated from the roof plate lineage and whose development is at least partially regulated by the rhombic lip (Broom et al., 2012), is an attractive candidate for orchestrating coordinated changes in cell fate through the secretion of a range of factors, including Shh (Huang et al., 2010), Igf2 (Lehtinen et al., 2011), retinoic acid (Yamamoto et al., 1996; Wilson et al., 2007) and thyroid hormone (Koibuchi, 2008). The choroid plexus may thus prove to be a factor in early cerebellar dysgenesis and offer a locus for understanding the coordinated diversification of glutamatergic and GABAergic neuronal subtypes during cerebellar evolution.

Conclusions

As with the study of many parts of the developing brain, specialised interests in specific phases of growth of subtypes of cells sometimes obscure a larger picture of coordinated programmes of development. These grander designs are often most apparent in the patterns of adaptation in evolution, where coherent regulation of developmental processes across time and scale are absolutely aligned. For this reason, the exploration of cerebellar development across a range of species will continue to be a valuable resource for generating perspective on development problems. For the cerebellum, the promise of a broad overview of how components of development are orchestrated seems particularly close due to its relatively simple circuit and small number of component cell types.

In this Review, we have discussed how familiar models of cell fate specification within the cerebellar territory have been revised as a result of increasingly sophisticated genetic approaches to deciphering the function of developmental genes. Thus, dorsoventral zones of cell specification do not constitute sites of lineage restriction, but rather sites of fate induction. The significance of progenitor movement between compartments *in vivo*, and its significance remain unclear. However, a common timetable for cell specification, independent of subtype (Yamada et al., 2014), hints at a common mechanism for coordinating the development and evolution of cell diversity. The identity of this coordinating signal remains an important issue for future research.

In an analogous manner that again points to homologies between seemingly diverse cell types, it has become clear that the secondary proliferative zones that emerge from precursor pools (the EGL, white matter stem cells and roof plate) all respond to a common signal: Shh. Thus, the elaboration of cerebellar structure brought about by the EGL is, in mammals, elegantly coupled to the number and perhaps diversity of interneurons. At the centre of this relationship, the early embryonic interactions that link choroid plexus and rhombic lip development (Broom et al., 2012) may graduate during development into an intra-ventricular signalling mechanism that coordinates different aspects of cerebellar development (Johansson et al., 2013). Whether the evolutionary emergence of the EGL was accompanied by the emergence of other secondary, transit-amplifying epithelia would provide an interesting perspective on this argument.

Perhaps the most subtle, though important, revision of conventional wisdom on cerebellar development is the demonstration that the cerebellar anlage is superseded by an isthmic anlage of Lhx9-positive extra-cerebellar nuclei (Green et al., 2014). Rhombomere 1 does not equate to the cerebellum, nor does FGF induce its development. Rather, the temporal dynamics of FGF signalling appear to affect a balance of cell production and are capable of generating a diversity of isthmic specialisations. Crucially, the human vermis, the genesis of which seems vital for a range of cognitive and affective behaviours, is a product of this process (Yu et al., 2013).

Finally, cerebellar functions beyond the traditional role of sensorimotor integration appear to rely on cerebellar communication with the cortex via the thalamus that depends explicitly on the dentate nucleus. Understanding the genetics underlying the production of distinct nuclei and their distinct projection patterns is an important future challenge. In this regard, the cerebellum is likely to remain at the forefront of developmental neuroscience.

Competing interests

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