THE CEREBELLUM: FROM DEVELOPMENT TO LEARNING

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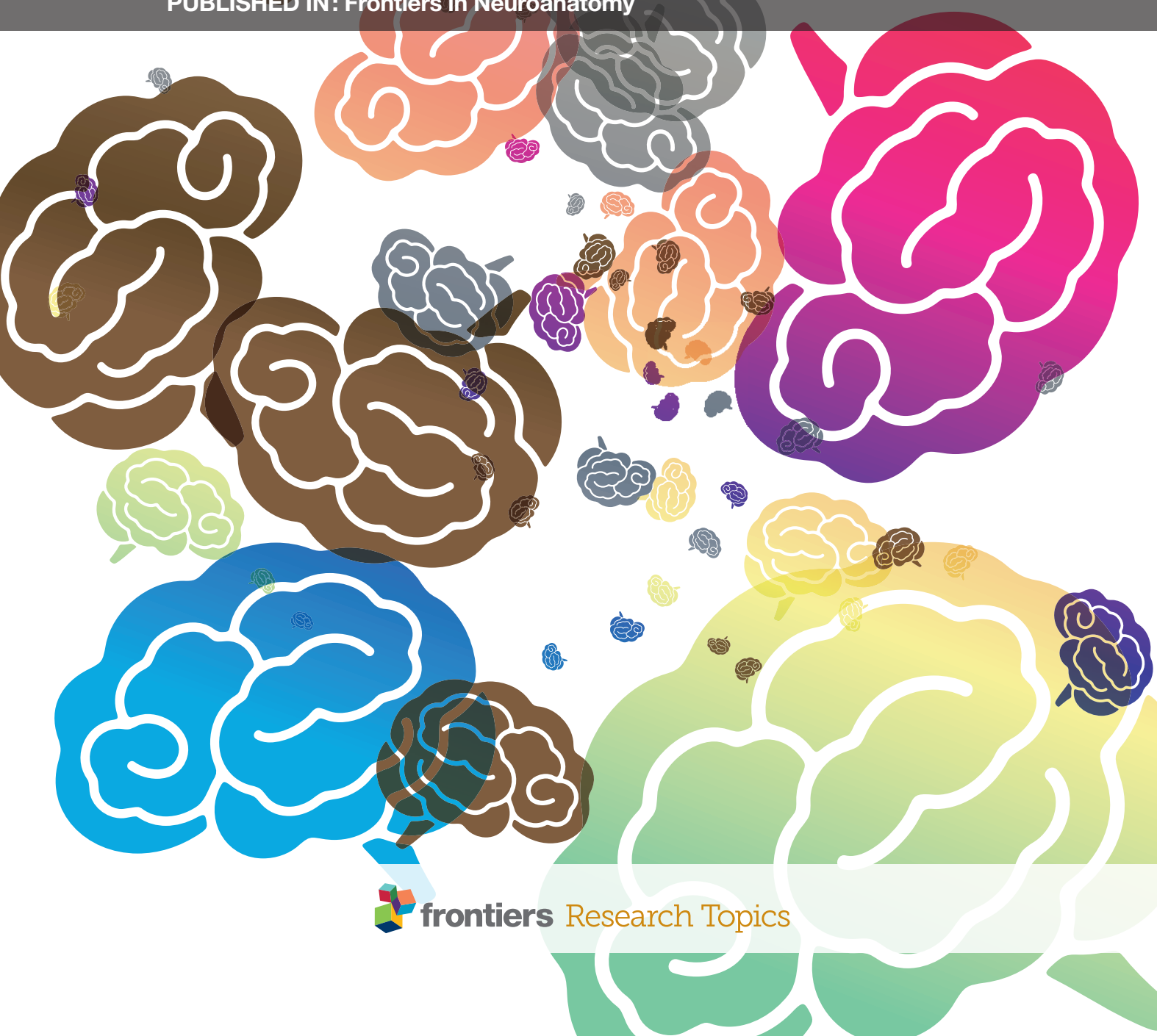

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THE CEREBELLUM: FROM DEVELOPMENT TO LEARNING

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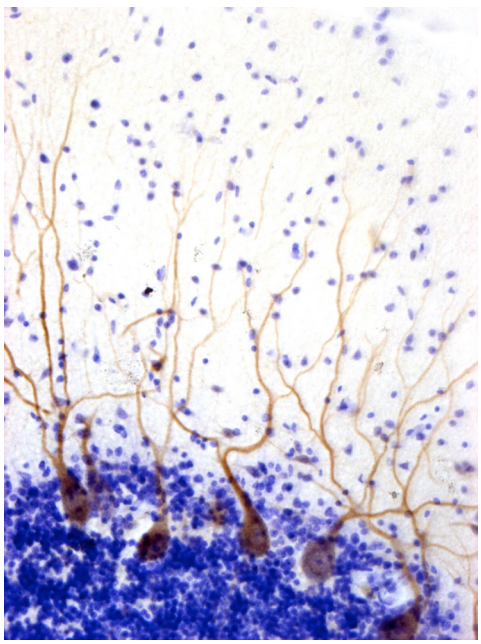

Human cerebellum cortex in a sagittal section, processed by anti-calbindin antibody (braun staining in Purkinje cells) and Nissl staining (violet color). Image by S. Martinez.

In this book, laboratory leaders on cerebellar neuroscience have revised the present body of knowledge about cerebellum anatomy and function. The trip throughout the cerebellar vineyard organization starts from the causal study of morphogenesis (that is, the molecular and cellular mechanisms underplaying form generation) to the molecular mechanism regulation cellular differentiation: Basson, Dusart, Hawkes, Martinez and Rosi's groups contributions. Then, neurodevelopmental anomalies associated with structural disorganization are revised in Jissendi and Batkovich's group reviews, describing and discussing pathogenic processes. Finally, functional mechanisms of cerebellar circuits involved in motor learning are revised by Delgado-Garcia and Armengol's group contribution.

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The cerebellum: from development to structural complexity and motor learning

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Keywords: cerebellum development, cerebellum and cognition, brain regionalization, Purkinje neuron, cerebellar regionalization, cerebellum circuits, opto-kinetic reflex, cerebellar interneurons

The cerebellum coordinates motor activities to be performed or that are already underway. In fact, it is very well known that cerebellar damage produces disturbance in movements and in body support. The relationship between cerebellum and motor learning was first suggested with the studies of Ramón y Cajal (1911), Dow and Moruzzi (1958), and Eccles et al. (1967). Dow and Moruzzi (1958) hypothesized that the cerebellum contributes to motor learning by determining how to perform accurate and correct movements (revised in Ito, 2002). Thereafter, numerous studies have been devoted to analyzing the role of the cerebellum in perceptive and cognitive processes. Thus, the essential contribution of Marr, localizing the site of motor learning in the cerebellar cortex (Marr, 1969), and the later application of Marr's theory to the classical conditioning (Albus, 1971), whose physiological basis is directly related to long-term depres-sion (LTD) mechanisms (Ito, 1989), defined the neuronal circuit involved in associative motor learning (revised by Porras-García et al., 2013). Although this appearance of agreement in these general aspects of cerebellar contribution to motor learning, the underlying cellular mechanisms of this contribution are far to be clear. Actually, recent reviews were published trying to get a consensus about the functional complexity that links the cerebellar intrinsic and extrinsic circuits with the motor coordination and learning; to conclude that there is still a lot of work to be done to get a precise idea about cerebellar cognitive function and the physiopathology of behavioral deficits in cerebellar dysfunctions (Ito, 2006, 2008; Manto et al., 2012). In this book, functional mechanisms of cerebellar circuits involved in motor learning are represented by the contribution of Dr. Jose Maria Delgado-Garcia's group (Sánchez-Campusano et al., 2012), where by modern electrophysiological methods they accurately analyzed the role of interpositus neurons in eyelid kinetics, demonstrating that antagonistic groups of deep cerebellar nuclei neurons are required for proper dynamic control of learned motor responses.

In parallel to the knowledge deficits on cerebellar function, cerebellar morphogenesis, which seems to be simple due to the repetitive myelo- and cyto-architecture along the whole organ, has revealed in the last two decades inspected complexities. First by the discovering of molecular heterogeneities in Purkinje cells; that is, new antigens (among then zebrins were pioneers) revealed a repetitive-stripe like structural organization of cerebellar Purkinje cells that reminded sagittal distribution of cortical afferences and efferences in the cerebellum. Then, probably for first time in vertebrates the molecular architecture reminded functional organization of neuronal circuits. Moreover, the expression patterns of these markers during development showed how Purkinje cells regulate the fundamental processes in the structural and functional development of the whole cerebellum. This was a seminal result to develop causal ontogenetic studies on the molecular control of cerebellar structure and function (reviewed in Dastjerdi et al., 2012; White and Sillitoe, 2013). Second, complexity also derived from experimental embryology approaches revealed a heterogeneous origin of cerebellar precursors, from at least three different domains: caudal mesencephalon, isthmus and rhombencephalon. Each domain originated different cerebellar regions: anterior and posterior vermis, as well as the cerebellar hemispheres, respectively (Martinez et al., 2013). Moreover we have strongly advanced in identifying the molecular mechanisms underlying internal cerebellar regionalization. The revisions from Martinez et al. (2013) and Basson and Wingate (2013) describe the embryology and morphogenesis of cerebellar anlage, which is controlled by different organizer regions and morphogenetic signals. The relation between neuroepithelial microdomains and Purkinje neurons specification to develop antigenic-defined stripes is extensively revised in Dastjerdi et al. (2012).

The contribution from Dr. Ferdinando Rossi laboratory (Leto et al., 2012) describes how neural progenitors of cerebellar GABAergic neurons have different origin, in relation to the neuronal character: (1) projection GABAergic neurons are originated from ventricular progenitors locally committed to their fate under cell autonomous mechanisms (Leto et al., 2012); which are consequence of positional information defined microdomains in the neuroepithelium (see Dastjerdi et al., 2012; Basson and Wingate, 2013; Martinez et al., 2013). (2) Conversely, GABAergic cerebellar interneuron progenitors are multipotent and sensitive to spatiotemporally patterned environmental signals that regulate the genesis of different categories of interneurons, in precise quantities and at defined times and places. Our friend Dr. Ferdinando Rossi passed away on January 24th, 2014, shortly after having made this contribution. His excellent and highly significant scientific legacy will continue to illuminate us in understanding the cerebellum.

Dr. Isabelle Dusart's group contribution (Dusart and Flamant, 2012) describes the strong structural changes that Purkinje neurons suffer during the two first postnatal weeks and the significant role of thyroid hormones in this process. The cerebellar alterations of hypothyroidism have been described in Dr. Manto and Dr. Jissendi paper (2913).

Barkovich (2012) revises the most frequent malformation patterns in humans and discusses about potential underling causal molecular and cellular mechanisms that operating during cerebellar development can explain the observed malformations. This revision is complemented by the contribution of Manto and Jissendi (2012) were cerebellar anomalies associated to genes regulating neural migration and synaptogenesis were revised, together with other noxious situations. Interestingly, pathogenic predictions developed from the molecular and genetic embryonic approaches (Basson and Wingate, 2013; Martinez et al., 2013) were clearly recognizable in the described clinical phenotypes.

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Congenital hypoplasia of the cerebellum: developmental causes and behavioral consequences

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Over the last 60 years, the spotlight of research has periodically returned to the cerebellum as new techniques and insights have emerged. Because of its simple homogeneous structure, limited diversity of cell types and characteristic behavioral pathologies, the cerebellum is a natural home for studies of cell specification, patterning, and neuronal migration. However, recent evidence has extended the traditional range of perceived cerebellar function to include modulation of cognitive processes and implicated cerebellar hypoplasia and Purkinje neuron hypo-cellularity with autistic spectrum disorder. In the light of this emerging frontier, we review the key stages and genetic mechanisms behind cerebellum development. In particular, we discuss the role of the midbrain hindbrain isthmic organizer in the development of the cerebellar vermis and the specification and differentiation of Purkinje cells and granule neurons. These developmental processes are then considered in relation to recent insights into selected human developmental cerebellar defects: Joubert syndrome, Dandy–Walker malformation, and pontocerebellar hypoplasia. Finally, we review current research that opens up the possibility of using the mouse as a genetic model to study the role of the cerebellum in cognitive function.

Keywords: cerebellum, development, defects, hypoplasia, genetics, function, behavior, autism spectrum disorders

INTRODUCTION

The cerebellum is an intriguing component of the central nervous system. From one perspective it is a famously simple neuronanatomical circuit constructed from a relatively few neuronal types and comprising a single uniform microarchitecture (Cajal, 1894; Eccles et al., 1967). However, the nature of the calculations performed by this circuit and its precise role in a variety of different neural functions has proved notoriously difficult to pin down. Despite the conserved nature of its core functional neuronal partnership, formed between granule cell axons and Purkinje cell dendrites, it is also clear that the cerebellum is employed as a neural "comparator" in different ways in different species (Meek, 1992; Barlow, 2002). From a predominantly proprioceptive and sensory role in fish, it has adopted more overt motor functions in mammals (Nieuwenhuys et al., 1998). In primates, including humans, a large proportion of the cerebellar cortex is in addition given over to interactions with regions of the cortex involved in cognition and judgment (Strick et al., 2009). The recruitment of a relatively unchanging core cerebellar circuitry into a variety of different functions both presents challenges in understanding its role in human disease but also great potential for the use of simpler model animal systems in solving these challenges.

Despite the uniformity of its cellular structure, the cerebellum is divided into clear anatomical divisions on the basis of a transverse fissures that separate lobes. These are folds in what is a continuous ribbon of neural circuitry that, in humans, would extend over a meter in anteroposterior length (Braitenberg and Atwood, 1958). A primary fissure divides the anterior from the posterior lobes, while a posterolateral fissure separates posterior lobe from a distinct flocculonodular lobe. Perpendicular to these, longitudinal, deep furrows partition the two cerebellar hemispheres (both with intermediate and lateral zones) from a central "vermis." While the flocculonodular lobe sits somewhat apart as a region with direct vestibular interactions (the "vestibulocerebellum"), the vermis, intermediate and lateral cerebellar hemispheres each predominantly target a different cerebellar nucleus that lies in the white matter beneath the cerebellar cortical layers. Thus for the majority for the cerebellum, the targeting of output of each nucleus determines the functional output of

Abbreviations: Ahi1, Abelson helper integration site 1; Apc, adenomatous polyposis coli; Atoh1, atonal homolog 1; BMI1, B lymphoma Mo-MLV insertion region 1 homolog; CASK, calcium/calmodulin-dependent serine protein kinase; Cep290, centrosomal protein 290 kDa; CHMP1A, charged multivesicular body protein 1a; EGL, external granule cell (germinal) layer; En1, engrailed 1; En2, engrailed 2; EXOSC3, exosome component 3; FGF, fibroblast growth factor; Foxc1, forkhead box C1; Gbx2, gastrulation brain homeobox 2; GCps, granule cell precursors; Gli, GLI-Kruppel family member; Ink4a, cyclin-dependent kinase inhibitor 2A, p16Ink4a; IsO, isthmus organizer; KAL1, Kallmann syndrome 1 sequence; LMX1B, Lim homeobox transcription factor 1 beta; Otx2, orthodenticle homeobox 2; Pax2, paired box gene 2; PC, Purkinje cell; Ptf1a, pancreas transcription factor 1 subunit alpha; r1, rhombomere 1; RARS2, arginyl-tRNA synthetase 2; Reln, Reelin; RL, rhombic lip; Shh, Sonic Hedgehog; Smo, smoothened; SUFU, suppressor of fused homolog; TGFβ, transforming growth factor beta; Tmem67, transmembrane protein 67; Tmem216, transmembrane protein 216; Tsc1, tuberous sclerosis complex 1; TSEN2, tRNA-splicing endonuclease subunit 2; TSEN34, tRNA-splicing endonuclease subunit 34; TSEN54, tRNA-splicing endonuclease subunit 54; vz, ventricular zone; Wnt1, wingless-type MMTV integration site family, member 1; ZIC1, zinc finger protein of the cerebellum 1; ZIC3, zinc finger protein of the cerebellum 3; ZIC4, zinc finger protein of the cerebellum 4.

the overlying cerebellar cortex. In mammals, the medial vestigial and interposed nuclei mainly target descending motor systems, channeling the output of the vermis and intermediate hemispheric zone (the "spinocerebellum"). By contrast, the lateral zone of the cerebellar hemispheres is chiefly linked via the dentate nucleus to the thalamus and hence the cerebral cortex (the "cerebrocerebellum").

These three major functional subdivisions of the cerebellum have been long been recognized and used to calibrate defects in developmental morphogenesis, many of which, as described below, have a prominent affect on the vermis (spinocerebellum). However, it should be clear from the above that functional consequences of developmental disorders may also depend on the degree of disruption to the formation of cerebellar nuclei and the precision of their inputs, which are difficult to assess. In addition, appreciation of cerebellar dysfunction is colored by the simple constraint that the cerebellum is best-known and understood in terms of its integration of proprioceptive information in the control of movement (Sherrington, 1906; Holmes, 1939). Typical symptoms of cerebellar dysfunction include dyssynergia (problems with measuring appropriate muscle force), dysmetria (improper interpretation of distance), ataxia (disordered movement), and dysdiadochokinesia (inability to perform rapidly alternating movements). Therefore, although appreciated for some time, relatively little attention has been given to the involvement of the cerebellar system in cognitive and emotional behaviors. This might reflect both the immediate usefulness of simple motor tests to diagnose cerebellar damage (Holmes, 1939), but also a focus on descending motor systems that corresponds to the expectations of loss of the vermis.

However, recent studies have highlighted the possibility that cerebellar defects might underlie some of the symptoms in subsets of patients diagnosed with neurodevelopmental disorders like autism spectrum disorders (ASD), attention deficit hyperactivity disorder (ADHD), and schizophrenia (Courchesne et al., 1988; Bauman and Kemper, 1994; Mostofsky et al., 1998; Palmen et al., 2004; Bottmer et al., 2005; Fatemi et al., 2012; Vaidya, 2012; Villanueva, 2012). As these conditions are clinically heterogeneous, it remains near impossible to consistently link specific neuroanatomical defects, in the cerebellum or otherwise, to distinct behaviors. However, as our ability to classify patients into more homogeneous phenotypic groups improves with ever more powerful imaging techniques (Smyser et al., 2011; Haubold et al., 2012), and next generation sequencing approaches allows the identification of genetic alterations (Coe et al., 2012), the possibilities of linking genetic alterations with specific cerebellar defects and behaviors becomes imminently feasible. These analyses extend to conditions characterized by vermal agenesis, emphasizing the need for a fuller understanding of how cerebellar output and underlying anatomy are reorganized in these conditions.

These advances are coupled with an increasing potential to interpret both anatomical and genetic phenotypes in terms of specific aspects of cerebellar development. This has been driven by substantial progress in understanding the origins of different cerebellar cell types and their interactions within the last 10 years. Fate-maps based on the genetic identity of different cell types, a molecular dissection of their interactions and new anatomical techniques to trace long range connections in the brain (Strick et al., 2009) have revealed the underlying pathways for cerebellum growth and patterning. In particular, distinct developmental pathways for neurons with the cerebellar cortex and deep nuclei imply that different populations will be affected in different ways depending on the location and timing of a given genetic disorder. These point the way to a future where specific connections and neuron populations can be systematically investigated in the context of human disorders. However, our understanding of the mechanisms whereby these genetic alterations cause specific cerebellar pathologies and the exact behavioral consequences of these cerebellar defects remain limited. These questions are best addressed in model systems that allow the accurate perturbation of specific genes and/or pathways, coupled with an in-depth analysis of developmental processes over time. The mouse has emerged as a valuable model for three critical regions: (1) powerful genetic tools available in the mouse have made it possible to accurately fate-map cells that share the same genetic ancestry and (2) dissect the function of a gene at different developmental time points and in different cell types or brain regions with high precision, and (3) behavioral tests have been developed that can be applied to determine the consequences of defined defects on specific behavioral endo-phenotypes. The development of innovative approaches to map brain connectivity in mice will add yet another powerful tool to the available kit (Lo and Anderson, 2011).

As the classification of brain pathologies with cerebellar involvement and known genetic associations have been reviewed extensively (see for example Barkovich et al., 2009), our aim is not to recapitulate these in the present article. Instead, we aim to outline some of the key developmental processes that typically go awry during cerebellar development and use well-understood examples from mouse genetic studies to illustrate how different developmental defects or signaling defects that arise at different developmental stages cause distinct structural abnormalities of the cerebellum. This discussion highlights significant gaps in our understanding of the mechanisms that underlie cerebellar malformation. Finally, we briefly outline the current understanding of cerebellar connectivity with the neocortex that might underlie its role in higher order function and speculate how defects in cerebellar connectivity might underlie behaviors associated with neurodevelopmental disorders such as autism. This final section highlights the potential limits of the mouse model as a means of understanding the full range of cerebellar developmental disorders, when the functional connections between cerebellum, thalamus, and cortex are not yet fully understood.

CONGENITAL CEREBELLAR DEFECTS: DEVELOPMENTAL MECHANISMS

Developmental defects of the cerebellum can be present as part of more complex developmental syndromes, in combination with other nervous system defects such as cortical hypoplasia and corpus callosum agenesis or more rarely, as isolated defects. Clinical classification of cerebellar defects is difficult and several classification schemes have been proposed, some of which are based on embryological and genetic considerations (Barkovich et al., 2009).

These classifications are important in order for the correct division of patients for treatment and further genetic studies to identify the genetic causes responsible for cerebellar anomalies. Before we discuss recent advances in the genetics of human cerebellar hypoplasia, we first provide an overview of the key developmental processes that control cerebellar growth and morphogenesis, and discuss pertinent studies in mouse mutants upon which most of the interpretation of human malformations is based.

OVERVIEW OF KEY STAGES IN CEREBELLAR DEVELOPMENT: INSIGHTS FROM THE MOUSE

The isthmus organizer (IsO)

The cerebellum is derived from the dorsal part of the most anterior segment of the hindbrain, rhombomere 1 (r1; Millet et al., 1996; Wingate and Hatten, 1999). Thus, any developmental defect that results in the failure to specify the anterior hindbrain or r1 itself, will inevitably result in cerebellar aplasia (Eddison et al., 2004) as might global defects in dorsal patterning mechanisms (Chizhikov et al., 2006). Early steps in brain development include the specification of neural tissue (neural induction), formation, and internalization of the neural tube (neurulation), and patterning of the neural tube. The latter process imparts positional identity to different compartments along the anterior–posterior neuraxis, a process primarily achieved through the formation of specialized signaling centers, also referred to as secondary organizers. Secondary organizers secrete growth factors that pattern the adjacent tissue through the induction of distinct patterns of gene expression on either side of the organizer, as a result of the presence of the differential expression of competence factors (Kiecker and Lumsden, 2012). The signaling center that divides and patterns the mesencephalon and r1 is the mid-hindbrain or IsO. Classic studies in a number of model organisms have shown that the key organizing molecule secreted by the IsO is fibroblast growth factor 8 (FGF8; Crossley et al., 1996; Martinez et al., 1999). The initiation of *Fgf8* expression at the IsO is dependent upon the transcription factor LMX1B (Lim homeobox transcription factor 1 beta), whereas the position of the IsO at the mid-hindbrain boundary is determined the mutually repressive activities of the homeobox genes *Otx2* (orthodenticle homeobox 2) anteriorly, and *Gbx2* (gastrulation brain homeobox 2), posteriorly (Joyner et al., 2000; Guo et al., 2007). Once established, a stable transcriptional and signaling network maintains gene expression at the IsO. Critical components of this regulatory network include the transcription factors PAX2 (paired box gene 2), EN1 (engrailed 1), EN2 (engrailed 2), and GLI3 (GLI-Kruppel family member 3) and signaling molecules FGF8, FGF17, WNT1 (wingless-type MMTV integration site family, member 1), and SHH (Sonic Hedgehog; Wittmann et al., 2009). Detailed fate-mapping studies in the mouse have located the progenitors of the medial cerebellar vermis to anterior r1 of the early embryo (Sgaier et al., 2005). The spatial organization of gene expression patterns of *Wnt1* and *Fgf8* in relation to the approximate progenitor domains of the vermis and hemispheres are represented in **Figure 1**.

Conditional gene deletion experiments in the mouse have proven to be an extremely powerful approach to dissect different requirements of key signaling pathways during cerebellar development (**Table 1**). The FGF and WNT signaling pathways are prime examples. Since the initial identification of *Fgf8* and *Wnt1* gene expression in cells at the IsO (Wilkinson et al., 1987; Crossley and Martin, 1995), various approaches to disrupt the function of these genes during cerebellar development have been employed. The germline deletion of *Fgf8* revealed an early function in gastrulation, such that the role of *Fgf8* in cerebellar development could not be investigated in these mutants (Meyers et al., 1998). The deletion of *Fgf8* specifically from the early IsO was found to result in the rapid cell death of all progenitors of the midbrain and cerebellum, identifying FGF as an essential survival factor cells in the mesencephalic(mes)/r1 region. The analysis of embryos homozygous for hypomorphic alleles of *Fgf8*, suggested that the maintenance of normal levels of FGF8 signaling was particularly important for the formation of medial cerebellar tissue (Chi et al., 2003). The requirement for high FGF signaling during vermis development was confirmed in mouse mutants where FGF signaling was specifically inhibited in the developing mes/r1 region shortly after the initiation of *Fgf8* expression in the IsO. Furthermore, the loss of vermis progenitors was found to be associated with roof plate expansion in anterior r1 (Basson et al., 2008; **Figures 1D,E**). A study by the Joyner lab has shown that the developmental stage at which *Fgf8* expression is disrupted is a key determinant of the severity of vermis hypoplasia; *Fgf8* deletion from the early (pre-E9.5) IsO cause severe vermis hypoplasia, whereas only mild hypoplasia in the anterior vermis resulted from *Fgf8* deletion between E9.5 and E11 (Sato and Joyner, 2009).

In the case of *Wnt1*, the germline deletion of *Wnt1* resulted in a similar phenotype to the early mes/r1-deletion of *Fgf8*, namely the absence of the midbrain and cerebellum by birth (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). A similar phenotype is observed upon the deletion of β-catenin using a *Wnt1-Cre* line (Brault et al., 2001). The cerebella of mice homozygous for a hypomorphic allele of *Wnt1* (*swaying, sw*) essentially represent phenocopies of FGF hypomorphic cerebella, by displaying a specific loss of the cerebellar vermis (Thomas et al., 1991; Louvi et al., 2003). Temporal requirements for WNT signaling have not been mapped as extensively as for FGF, but the deletion of β-catenin after E12.5 using Nestin-Cre, resulted in cerebellar vermis hypoplasia defects similar to $Wnt1^{sw/sw}$ mutants (Schuller and Rowitch, 2007). This observation suggests that the requirement for WNT/β-catenin signaling during vermis development and midline "fusion" is later, or extends over a longer time window than the requirement for FGF signaling. Taken together, these studies indicate that the cerebellar vermis that develops from tissue in anterior r1 that is exposed to the highest levels of FGF and WNT for the longest time has the strictest requirement for these signals during development.

In keeping with this general theme, mice deficient in *En1*, the first engrailed homeobox gene to be expressed during cerebellar development, results in cerebellar vermis aplasia (Wurst et al., 1994). A substantial number of mice with conditional deletion of *En1* after E9 exhibit normal cerebella, confirming the importance of early *En1* expression (Sgaier et al., 2007).

The role of the SHH pathway in postnatal cerebellar development is well-understood (see below). However, recent studies have provided evidence for important roles for *Gli3* at the IsO,

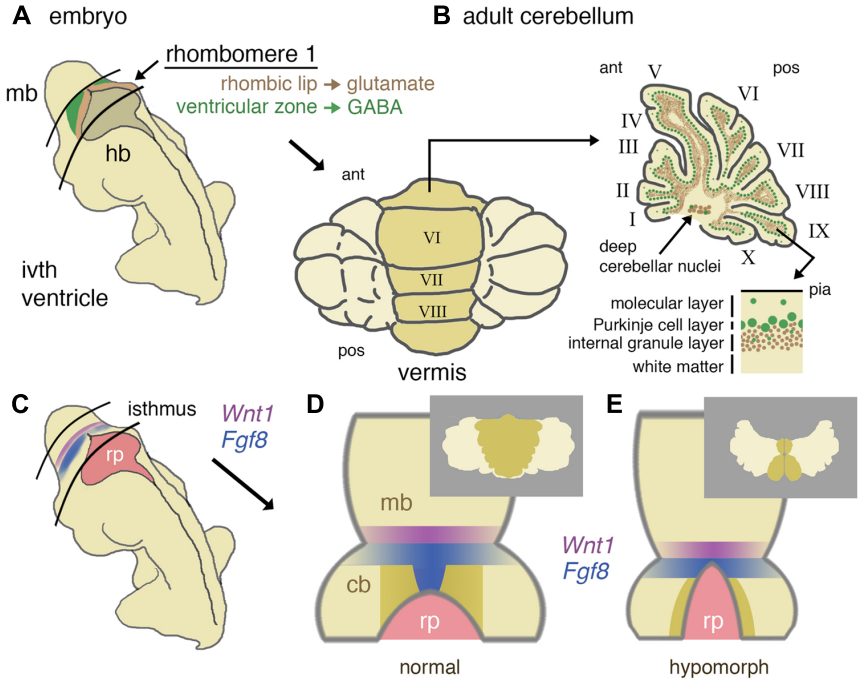

FIGURE 1 | Developmental origins of the mouse cerebellum and the role of isthmic gene expression in patterning the vermis. (A) Schematic representation of a mid-gestation embryo showing the location of derivatives of rhombomere 1. The ventricular layer (green) and rhombic lip (brown) of dorsal rhombomere 1 give rise to all GABA-ergic and glutamatergic cells of the cerebellum, respectively. **(B)** In a dorsal (posterior) view, the adult cerebellum is characterized by a central (darker shaded) vermis running anterior (ant) to posterior (pos). A uniform layering of cell types can be found throughout the vermis and more lateral hemispheres (shown in schematic parasagittal section), with GABA-ergic and glutamatergic differentially distributed in a later-specific manner: the molecular layer is largely reserved for the interaction of Purkinje cell dendrites and granule cell axons with sparse basket and stellate inhibitory interneurons. The Purkinje cells layer separates the molecular layer from an internal granule cell layer that contains a population of inhibitory Golgi cells. Deep cerebellar nuclei (GABA-ergic and glutamatergic neurons) lie within the white matter. **(C)** Schematic diagram showing the location of the isthmus organizer at the midbrain/hindbrain boundary with respect to the fourth ventricle roof plate (rp) and the expression domains of *Wnt1* (purple) and *Fgf8* (blue). **(D)** Dorsal schematic view of the isthmus region showing with darker shading the approximate region where progenitors of the cerebellar vermis reside, as based on inducible fate-mapping studies (Sgaier et al., 2005). The translation of this dorsal rhombomere 1 territory into adult vermis is shown **inset**. **(E)** Altered morphology of the isthmic region and reduced cerebellar size in a hypomorph with an altered function of the isthmic organizer due to diminished FGF signaling. Loss of vermis progenitors is concomitant with the expansion of the roof plate (adapted from Basson et al., 2008). The consequences for vermal morphogenesis in the adult are shown **inset**.

consistent with *Gli3* as a regulator of dorsal neural tube cell fates. Deletion of *Gli3* results in higher *Fgf8* expression and the expansion of the IsO (Aoto et al., 2002; Blaess et al., 2008). Recently, the conditional deletion of the SHH regulator, SUFU (suppressor of fused homolog), was shown to cause hyperplasia and disorganization of the IsO and mes/r1 regions. These early defects were also associated with ectopic and disorganized *Fgf8* expression at the IsO. Interestingly, these SUFU mutants exhibited cerebellar vermis hypoplasia that was almost entirely rescued by the constitutive expression of the GLI3 repressor (GLI3R) form, suggesting that this defect was primarily caused by the failure to generate GLI3R (Kim et al., 2011).

An important prediction of these studies in the mouse is that many human cerebellar disorders with strong cerebellar vermis involvement are likely to be caused by the disruption of IsO function during the earliest stages of cerebellar development. This proposition will be discussed further in Section "Mechanistic Insights into the Causes of Human Cerebellar Defects" in the context of recent findings on the mechanisms underlying human cerebellar malformations.

Establishment of progenitor zones and neurogenesis

After initial patterning and growth of r1 to form the cerebellar anlage, neurogenesis is initiated in two distinct germinal centers, the ventricular zone (VZ) and rhombic lip (RL). All cerebellar neurons and glia as well as progenitors that populate a number of extracerebellar nuclei are born within these germinal zones (**Figures 1A,B** and **2A**). Evidence that the production of different neuronal lineages is spatially restricted during cerebellar development comes from loss-of-function and lineage tracing experiments in the mouse. Ben-Arie et al. (1997) first demonstrated that the loss of *Atoh1* (atonal homolog 1), a gene specifically expressed in the RL, resulted in the failure to form an external germinal layer (EGL) and EGL-derived granule cells (**Figure 2B**).

The rhombic lip. Genetic fate-mapping studies and *Atoh1* loss-of-function studies have shown that progenitors of all excitatory glutamatergic neurons of the cerebellum are generated within the upper RL (Machold and Fishell, 2005; Wang et al., 2005). Defects in the formation or induction of the RL or the specification of granule cell progenitors (GCps) are predicted to result in severe cerebellar

Table 1 | Examples of mouse models that have informative cerebellar phenotypes and characterized developmental defects.

Mouse model	Developmental defects	Cerebellar phenotype	Comments
En1$^{cre/+}$;*Fgf8*$^{flox/flox}$ (conditional *Fgf8* deletion in mes/r1)	Programmed cell death of mes/r1 progenitors (Chi et al., 2003)	Agenesis	Germline *Fgf8* loss or hypomorphic mutations are embryonic lethal. Conditional gene deletion approaches have revealed different temporal and quantitative requirements for FGF signaling in vermis vs. hemisphere formation.
En1$^{cre/+}$;*Fgfr1*$^{flox/flox}$ (conditional *Fgfr1* deletion in mes/r1)	Defect in specification and/or expansion of vermis progenitors (Trokovic et al., 2003)	Vermis a/hypoplasia	
En1$^{cre/+}$;*Spry2GOF;Fgf8+/–* (reduction of FGF signaling specifically in the mes/r1 region)	Cell death in mes, failure to specify inferior colliculus (IC), defect in specification and/or expansion of vermis progenitors, roof plate expansion (Basson et al., 2008)	Vermis a/hypoplasia	
Wnt1–/–	Loss of mes/r1 progenitors (McMahon and Bradley, 1990)	Agenesis	Vermis and hemispheres have different quantitative and temporal requirements for WNT signaling, similar to FGF
Wnt1$^{sw/sw}$ (*Wnt1* hypomorph)	Defect in specification and/or expansion of vermis progenitors, roof plate expansion (Thomas et al., 1991; Louvi et al., 2003)	Vermis a/hypoplasia	
Ahi1–/–	Expanded roof plate; reduced WNT signaling and progenitor expansion in the medial vermis (Lancaster et al., 2009)	Vermis hypoplasia	*AHI1* mutations cause Joubert syndrome (Dixon-Salazar et al., 2004; Ferland et al., 2004)
Tmem67–/–	Expanded roof plate	Vermis hypoplasia	*TMEM67* (*MKS3*) mutations cause Joubert syndrome (Baala et al., 2007)
En1–/–	Failure to specify IC, defect in specification and/or expansion of vermis progenitors (Wurst et al., 1994)	Vermis a/hypoplasia	
En2–/–	Altered cerebellar foliation and growth. *En2* controls timing of fissure formation together with *En1* (Cheng et al., 2010; Orvis et al., 2012)	Subtle foliation defects (Joyner et al., 1991)	*EN2* polymorphisms linked to autism (Gharani et al., 2004)
Gbx2–/–	Failure of r1 specification and transformation into mes (Millet et al., 1999)	Cerebellar agenesis	No human mutations identified yet
Gbx2$^{neo/neo}$ (Gbx2 hypomorph)	Defects in anterior r1 (Waters and Lewandoski, 2006)	Vermis hypoplasia	
En1$^{Otx2/+}$ (misexpression of *Otx2* through the mes/r1 region)	Caudal repositioning of IsO, expansion of mesencephalon at the expense of anterior r1 (vermis progenitors; Broccoli et al., 1999)	Vermis aplasia	
Lmx1b–/–	Defect in establishing and maintaining the IsO (Guo et al., 2007)	Vermis agenesis	

(Continued)

Table 1 | Continued

Mouse model	Developmental defects	Cerebellar phenotype	Comments
Lmx1a−/−	Smaller roof plate	Mild cerebellar hypoplasia	
Lmx1a−/−;Lmx1b$^{cko/-}$	Very small roof plate (Mishima et al., 2009)	Severe cerebellar hypoplasia	
Ptf1a−/−	Ventricular zone defects (Hoshino et al., 2005; Pascual et al., 2007)	Severe cerebellar hypoplasia	Cerebellar agenesis in humans with homozygous *PTF1A* loss of function (Sellick et al., 2004)
Atoh1−/−	Failure to form EGL (Ben-Arie et al., 1997)	Severe cerebellar hypoplasia	*Atoh1* is required for *Shh*-responsiveness and GCp proliferation (Flora et al., 2009)
L7-Cre;Shh$^{flox/flox}$ (conditional deletion of *Shh* from PCs)	Reduced GCp proliferation (Lewis et al., 2004)	General cerebellar hypoplasia	
hGFAP-Cre;Smo$^{flox/flox}$ *hGFAP-Cre;Kif3a*$^{flox/flox}$ Conditional deletion of *Smo* and *Kif3a* from GCp lineage	Reduced GCp proliferation (Spassky et al., 2008)	General cerebellar hypoplasia	Similar phenotypes have been reported upon deletion of other downstream effectors of SHH signaling, e.g., *Gli2* (Corrales et al., 2004, 2006)
Zic1−/−;Zic4−/−	Reduced GCp proliferation, unknown developmental causes for anterior vermis defects (Blank et al., 2011)	Cerebellar hypoplasia	*ZIC1+/-;ZIC4+/-* haploinsufficiency linked to Dandy–Walker malformation (Grinberg et al., 2004; Tohyama et al., 2011)
Foxc1−/−	Enlarged roof plate, disorganized rhombic lip, loss of Atoh1 expression in medial cerebellum, reduced *Tgfb1*, *Cxcl12*, *Bmp2*, and *Bmp4* expression (Aldinger et al., 2009)	Vermis a/hypoplasia	*FOXC1* deletions or duplications associated with cerebellar hypoplasia and Dandy–Walker malformation in humans (Aldinger et al., 2009)
Pcp2-cre;Tsc1$^{flox/flox}$ (Purkinje cell-specific deletion of *Tsc1*)	Apparent normal cerebellar development (Tsai et al., 2012)	Postnatal Purkinje cell loss and increased dendritic spine density (Tsai et al., 2012)	*TSC1* or *TSC2* mutations associated with cerebellar tubers (tuberous sclerosis) and autism (Eluvathingal et al., 2006)
Reln	Defects in PC migration and secondary GCp expansion (Mariani, 1982; Miyata et al., 2010)	Severe cerebellar hypoplasia (Magdaleno et al., 2002)	*RELN−/−* patients have cerebellar hypoplasia (Hong et al., 2000)
Vldlr−/− and *Apoer2−/−*(genes encoding Reln receptors)	Abnormal PC migration and dendritic arborization (Trommsdorff et al., 1999)	General cerebellar hypoplasia	*VLDLR−/−* patients have pontocerebellar hypoplasia and quadrupedal locomotion (Ozcelik et al., 2008)

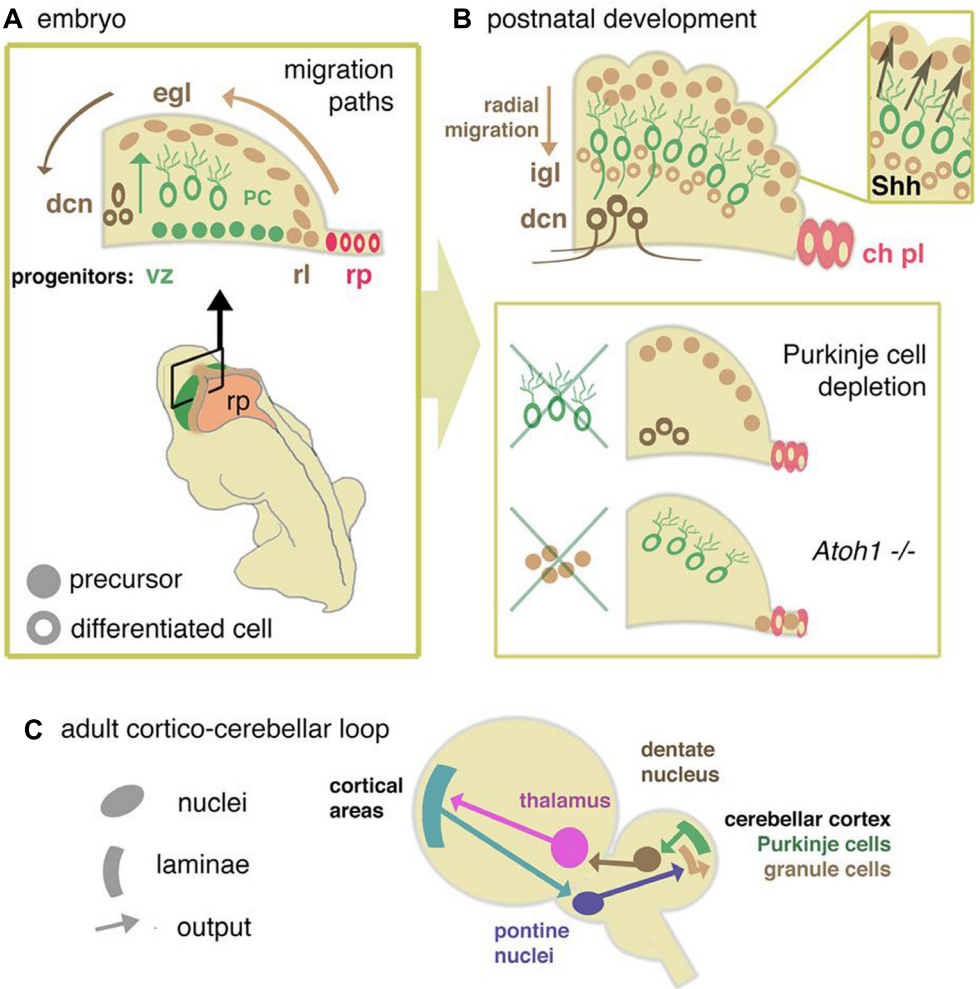

FIGURE 2 | Cellular development of cerebellum and components of the re-entrant cortico-cerebellar loop. (A) Schematic cross sectional view through the anlage of the cerebellum showing the relationship between rhombic lip (rl), ventricular zone (vz), and roof plate (rp) of the fourth ventricle. Adult morphological layering (**Figure 1**) is the product of two major migration pathways. The vz gives rise to GABA-ergic radially dispersed Purkinje cells (**oc**). The rl generates glutamatergic deep cerebellar nucleus (**dcn**) neurons and granule cell precursors of the external germinal layer (**egl**), which migrate tangentially from the rl in sub-pial streams. **(B)** Postnatally, the egl proliferates under the influence of Purkinje cell-derived Shh. Postmitotic glutamatergic granule cells migrate radially from the EGL to internal granule layer (**igl**). In mutants where Purkinje cells are deleted, or in which Shh is depleted, or where egl formation is suppressed (as in the *Atoh1* knockout mouse), cerebellum growth is reduced. Disruptions in signals from the overlying mesenchyme, directly or through affecting signaling from the roof plate, may modulate the responsiveness of the egl to mitogens, thus abrogating its expansion. **(C)** From data derived in the primate (Strick et al., 2009) a general model for a mammalian circuit would propose that cortico-cerebellar closed loops modulate cortical activity for a number of different motor and non-motor cortical areas. Without presupposing details of cortical areas in the mouse, cortical activity would be anticipated to feed into the cerebellar circuit via the pontine nucleus, a derivative of the rhombic lip of the hindbrain. Cerebellar output via dentate nucleus neurons would then feed back to the cortical areas via the thalamus.

hypoplasia due to the absence of this rapidly proliferating transit amplifying cell population during postnatal development (see Progenitor Cell Migration, Proliferation, and Differentiation). The mechanisms required for the induction and functionality of the RL are being elucidated. A number of signaling pathways, including the TGFβ (transforming growth factor beta) and Notch pathways and signaling from the roof plate (Alder et al., 1996; Chizhikov et al., 2006) are implicated in the induction of the RL. Cell production from the RL appears to involve an iterative induction of *Atoh1* in successive waves of migratory derivatives (Machold et al., 2007; Broom et al., 2012).

In addition to GCps, the *Atoh1*-positive RL also gives rise to neurons that populate the deep cerebellar and extracerebellar nuclei; these include both glutamatergic and cholinergic neurons (Machold and Fishell, 2005; Wang et al., 2005; Wingate, 2005). Moreover, the RL extends into the hindbrain where it generates neurons that participate in a number of defined circuits including mossy fiber inputs to granule cells via the pons (Rodriguez and Dymecki, 2000; Rose et al., 2009). This raises the possibility that defects across the extent of the cerebellar and hindbrain RL could be the cause of conditions, such as pontocerebellar hypoplasia (see below) where multiple distributed elements of the cerebellar

systems are disrupted. Developmental defects affecting this progenitor zone and its descendants might have far-reaching effects on cerebellar connectivity (**Figure 2A**). In particular, these discoveries also point to a time window of sensitivity to developmental damage that might target deep cerebellar nuclei but leave later born granule cell derivatives untouched. Such a window of potential vulnerability to intrinsic or extrinsic damage to the embryo might have selective effects on cerebellar function (in particular connectivity) that are not necessarily correlated with substantial reduction in cerebellar size.

Ventricular zone. Genetic fate-mapping of cells in the cerebellar VZ, demonstrated that all GABA-ergic neurons, including Purkinje, Golgi, basket, and stellate cells, as well as small GABA-ergic neurons of the deep cerebellar nuclei are derived from this region (Hoshino et al., 2005; Sudarov et al., 2011; **Figure 2A**). Compared to the number of glutamatergic granule neurons in the adult cerebellum, the contribution of GABA-ergic neurons to the over-all size of the cerebellum is relatively minor. Thus, defects in the generation of GABA-ergic neurons are not expected to result directly in significant cerebellar hypoplasia. However, as we discuss in the next section, VZ-derived Purkinje cell progenitors are the primary source of mitogen to GCps in the EGL. Thus, the absence or mislocalization of Purkinje cells due to VZ defects could be responsible for cerebellar hypoplasia owing to a deficit in GCp proliferation and postnatal cerebellar growth (**Figure 2B**). Indeed, Hoshino et al. (2005) showed that the disruption of the Ptf1a (pancreas transcription factor 1 subunit alpha) gene by transgenic insertion resulted in the compete loss of GABA-ergic lineages in the cerebellum and severe cerebellar hypoplasia. It is important to note that Bergmann glia are also derived from the VZ. As these cells form the scaffold that guides the radial migration of neuronal progenitors, defects in the generation or differentiation of these cells could also be responsible for the failure of Purkinje cell migration (see Progenitor Cell Migration, Proliferation, and Differentiation).

Finally, evidence for interaction between progenitor zones comes from the analysis of cell fate upon the deletion of *Ptf1a* and *Atoh1*. Pascual et al. (2007) showed that VZ-derived progenitors that develop in the absence of the transcription factor PTF1A, invade the EGL and adapted glutamatergic fates reminiscent of RL-derived progenitors, indicating that PTF1A actively represses glutamatergic fate to maintain GABA-ergic fate determination. Similarly, Rose et al. (2009) found that deletion of *Atoh1* results in RL cells entering the roof plate, indicating that ATOH1 activity suppresses the adoption of this non-neuronal fate. This is reminiscent of the mutual inhibitory interactions that specify progenitor domains within the spinal cord (reviewed by Wilson and Maden, 2005). Defects in cross-regulation, or in the formation or maintenance of cerebellar germinal zones may result in cerebellar hypoplasia by directly disrupting the formation of cerebellar neurons, or by undermining subsequent interactions that lead to the massive expansion of the granule cell precursor pool in the EGL.

Progenitor cell migration, proliferation, and differentiation

Tissue growth in the developing embryo has to be tightly regulated to allow the coordinated expansion of different cell types. Coordinated growth requires communication between two or more closely apposed tissue or cell layers. Perhaps the best-known example is the orchestration of epithelial growth and morphogenesis through epithelial–mesenchymal interactions. Postnatal cerebellar growth is regulated in a similar manner. Rapid cerebellar growth is primarily driven by the proliferation of GCps in the EGL, a process largely coordinated by a layer of Purkinje neurons under the surface of the cerebellum (Hatten and Heintz, 1995). As we have discussed, the failure to specify Purkinje neurons is associated with severe cerebellar hypoplasia. After their birth in the VZ, Purkinje neuron progenitors migrate along radial glia toward the pial surface of the cerebellar anlage. Genetic defects that disrupt the glial scaffold, or the production of signals and cell-intrinsic mechanisms that control Purkinje cell migration result in various degrees of cerebellar hypoplasia (**Figure 2B**). In addition, cell migration defects resulting in the ectopic localization of Purkinje cells are likely to underlie many examples of cerebellar heterotopias (Yang et al., 2002).

One of the central pathways linked to GCp proliferation and differentiation is the SHH pathway. Immature Purkinje cells secrete SHH and that the proliferation of GCps is critically dependent on SHH signaling (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). In mouse, conditional deletion of *Shh* from PCs (Purkinje cells) or SHH signal transduction components like *Smo* (smoothened), *Gli1*, and *Gli2* from GCps have all been shown to result in defects in GCp proliferation and cerebellar hypoplasia (Lewis et al., 2004; Corrales et al., 2006; Spassky et al., 2008). Disrupting PC migration or differentiation result in similar phenotypes (Hatten, 1999). For example, mice homozygous for the reeler allele, $Reln^{rl/rl}$, exhibit severe cerebellar hypoplasia (Magdaleno et al., 2002). In the absence of Reelin, PCs fail to organize in the form of a Purkinje plate under the pial surface of the E14.5 cerebellum (Mariani et al., 1977; Miyata et al., 1997). These observations suggest that the primary cause of cerebellar hypoplasia associated with defects in Reelin signaling is the failure of SHH-expressing PCs to reach their appropriate position underneath the EGL where they provide a proliferative SHH signal.

It is important to note that cerebellar hypoplasia caused by defects in SHH signaling affects the (primarily postnatal) proliferation of GCps in the vermis and hemispheres equally, resulting in a phenotype that differs significantly from early IsO defects with disproportionally hypoplastic vermis.

Conditional manipulation of signaling pathways that function during early cerebellar development have revealed additional functions during later stages of cerebellar development. Again, the WNT and FGF pathways provide good examples of this principle. Reduced WNT signaling during early development result in cerebellar defects typical of reduced IsO function, i.e., vermis aplasia (see The Isthmus Organizer). WNT/β-catenin signaling is also active at later stages of cerebellar development, particularly in the germinal zones and Bergmann glia (Selvadurai and Mason, 2011). The role of WNT/β-catenin signaling at later developmental stages has been investigated more recently. Several groups have reported that increased β-catenin signaling can alter the proliferation and differentiation of neuronal progenitors in the developing cerebellum. Lorenz et al. (2011) showed that activation of WNT/β-catenin signaling by deletion of the *Apc* (adenomatous polyposis coli)

gene or stabilization of β-catenin in GCps inhibited their proliferation and enhanced their differentiation. As a consequence, these mice exhibited cerebellar hypoplasia and ataxia. The Wechsler-Reya group confirmed the potent inhibition of GCp proliferation and cerebellar hypoplasia as a result of hyperactivation of β-catenin in GCps, but found additionally that the proliferation of GABA-ergic progenitors in the VZ was significantly increased when signaling was increased in this progenitor zone (Pei et al., 2012). The observation of differential effects of increased WNT signaling on progenitors in different anatomical locations is also important in understanding the origins of childhood tumors of the cerebellum, medulloblastoma. Medulloblastoma subtypes characterized by activating mutations in WNT pathway genes are most likely to originate from the lower, i.e., non-cerebellar RL and not from cells in the EGL (Gibson et al., 2010), in agreement with the finding that WNT activation inhibits cell proliferation in the EGL.

Similar complexities have been observed upon altering FGF signaling during cerebellar development. Increased FGF signaling upon deletion of Sprouty genes, which encode FGF antagonists, have opposite effects on cerebellar growth depending on the time of deletion. Upregulated FGF signaling during early development is associated with the expansion of vermis progenitors in anterior r1 and an expansion of the width of the vermis. Increased FGF signaling during late embryonic and postnatal stages on the other hand is associated with abnormalities in Bergmann glial and Purkinje cell differentiation, reduced SHH production by these cells as well as an inhibitory effect on the responsiveness of GCps to SHH. As a consequence of these changes, GCps prematurely exit the cell cycle and differentiate, leading to general cerebellar hypoplasia (Yu et al., 2011).

These observations provide a window on some of the complexities of understanding the role of particular pathways in cerebellar development. Not only does one pathway have multiple functions at different developmental time points, the effects of deregulated signaling in one neuronal progenitor subpopulation can be vastly different from another. In the light of these observations, any attempt to infer mechanistic explanations for cerebellar phenotypes from observations made in unrelated cell types or simplified *in vitro* cultures have to be interpreted with considerable caution. Although these studies are useful as initial proof-of-principle studies, validation and analysis in the appropriate cell types in an intact developing cerebellum are essential.

MECHANISTIC INSIGHTS INTO THE CAUSES OF HUMAN CEREBELLAR DEFECTS

Insights gained from studies in model organisms have greatly improved our ability to identify the underlying processes responsible for many congenital cerebellar anomalies that affect the human population. In this section, we aim to demonstrate some of these new insights by discussing our current understanding of two major syndromes associated with cerebellar hypoplasia: Joubert syndrome and Dandy–Walker malformation. We contrast the remarkable progress that has been made in our understanding of these syndromes with pontocerebellar hypoplasia, a group of conditions for which candidate genes have been identified but little mechanistic insights are available.

Disrupted WNT signaling in Joubert syndrome

Joubert syndrome is characterized by cerebellar vermis hypoplasia and abnormal superior cerebellar peduncles and associated with both movement disorders and mental retardation (Joubert et al., 1999). Joubert syndrome has been the subject of intense study by human geneticists and more than 20 disease-associated genes have been identified to date (Valente et al., 2013). All these genes appear to encode proteins that are associated with the assembly and biology of cilia, and Joubert syndrome can therefore be classified within the larger group of ciliopathies, alongside Bardet–Biedl and Meckel syndromes (Brancati et al., 2010).

Despite the wealth of human genetics data and direct links to ciliary assembly and function, the cilia-dependent developmental pathways disrupted in Joubert syndrome are poorly understood. A recent study by the Gleeson group provided important insights into the causes of cerebellar phenotypes in Joubert syndrome caused by mutations in *AHI1* (Abelson helper integration site 1). First, they showed convincingly that vermis hypoplasia in *Ahi1−/−* mouse mutants is associated with an expanded roof plate and a vermis midline "fusion" defect (Lancaster et al., 2011). This cerebellar midline defect phenocopies the vermis agenesis characteristic of mutants with reduced IsO function like $Wnt1^{sw/sw}$ and FGF hypomorphs (Louvi et al., 2003; Basson et al., 2008). From studies in the mouse, we would hypothesize that the apparent "fusion" defect in these mutants is primarily caused by the failure of vermis progenitors in the anterior cerebellar anlage to expand, a defect that is likely associated with the abnormal expansion of roof plate tissue (Basson et al., 2008). WNT signaling was not measured in *Ahi1−/−* embryos at early stages when the IsO is functional (E9.5–E12.5) and roof plate expansion at the expense of the expansion of vermis progenitors has been shown to take place in FGF pathway mutants. However, WNT signaling was diminished in the medial cerebellar anlage at E13.5, which correlated with reduced proliferation of vermis progenitors adjacent to the midline. Finally, WNT/β-catenin activation by LiCl treatment at E12.5 and E13.5 partially rescued the proliferation defect and midline "fusion," confirming reduced WNT signaling as an important pathogenic mechanism in Joubert syndrome (Lancaster et al., 2009). A similar, yet milder phenotype is also reported in *Cep290−/−* animals. Although WNT signaling was not investigated in the *Cep290* (centrosomal protein 290 kDa) mutants, the data suggest that a WNT signaling defects might also underlie Joubert syndrome caused by mutations in Joubert-associated genes other than *AHI1*. This study clearly implicates deregulated WNT signaling as a mechanism in Joubert syndrome.

Abdelhamed et al. (2013) recently reported some interesting observations implicating the Joubert syndrome gene, *Tmem67* (transmembrane protein 67) in the regulation of SHH and WNT signaling. The authors report a significant expansion of the fourth ventricle roof plate in *Tmem67−/−* embryos, a phenotype consistent with a disruption of early IsO function and vermis hypoplasia/aplasia. Based on the studies discussed so far, one might predict that reduced WNT signaling in these mutants may lead directly to reduced activity of the IsO, early loss of vermis progenitors, or reduced proliferation of cells at the midline, akin to the hypothesis to explain vermis defects in *Ahi−/−* embryos. Indeed, the authors present data to suggest that WNT/β-catenin signaling

is reduced in *Tmem67−/−* embryonic fibroblasts from embryos with Joubert syndrome-like phenotypes. They also report reduced *Shh* expression in the ventral neural tube. It will be important to examine WNT and SHH signaling in mes/r1 in *Tmem67* mutants, in order to draw direct conclusions as to the involvement of these pathways in cerebellar development at these critical early developmental stages. Taken together, these recent studies suggest that several signaling pathways might be affected as a result of defective ciliary function and that the cerebellar vermis phenotype in Joubert syndrome is due to the deregulation of signaling pathways required for early IsO function and vermis expansion.

In addition to the well-known WNT and SHH pathways, the non-canonical WNT-planar cell polarity (PCP) pathway has also been implicated in the pathogenesis of Joubert syndrome. Knockdown or mutation of *Tmem216* (transmembrane protein 216), a gene mutated in Joubert and Meckel syndromes, resulted in ciliogenesis defects, and impaired centrosome docking to cilia due to the mislocalization of hyperactivated RhoA. Disheveled1 (Dvl1) phosphorylation in response to *Tmem216* knockdown and mild PCP phenotypes were observed in zebrafish (Valente et al., 2010). The role of WNT-PCP pathway during cerebellar morphogenesis has not been investigated and the developmental processes that might be sensitive to disrupted WNT-PCP signaling remains an important question to address in cerebellar development.

Although the critical roles of FGF signaling during cerebellar development are well-established in model organisms, FGF signaling defects have not been linked directly to cerebellar vermis defects in humans. This might be due to the fact that FGF hypomorphic mutations that reduce FGF signaling sufficiently to cause a cerebellar vermis phenotype, are also detrimental to the development of many other organs, such that foetuses carrying these mutations will not survive past birth. Two groups have reported rare patients with Kallmann syndrome and partial cerebellar vermis a/hypoplasia, initially diagnosed as Dandy–Walker malformation (Ueno et al., 2004; Aluclu et al., 2007). Mutations in the coding sequence of *KAL1* (Kallmann syndrome 1 sequence) was excluded by Ueno et al. in their study, leaving the possibility that mutations in other Kallmann syndrome-associated genes that affect FGF signaling might be responsible.

Dandy–Walker malformation

Dandy–Walker malformation of the cerebellum is diagnosed upon the identification of vermis hypoplasia (Brodal, 1945), rotation of the vermis away from the brain stem and an enlarged posterior fossa. Behavioral pathology can include motor deficits consistent with cerebellum damage and, in 50% of patients, intellectual impairment that has been tentatively correlated with the degree of loss of vermal lobulation (Boddaert et al., 2003). Grinberg et al. (2004) identified the linked *ZIC1* (zinc finger protein of the cerebellum 1) and *ZIC4* (zinc finger protein of the cerebellum 4) genes on 3q24 as candidate genes, an observation since confirmed by other groups (Tohyama et al., 2011). Experiments in *Zic1−/−;Zic4−/−* mouse models indicated that these genes were required for the full responsiveness of GCps to SHH. *Zic1−/−;Zic4−/−* GCps correspondingly showed reduced proliferation and general cerebellar hypoplasia. These cerebella also showed the loss of the anterior folium in the vermis that was specifically due to the loss of *Zic1*, which is uniquely expressed in the VZ. Taken together, this study links *Zic1* and *Zic4* to the SHH pathway and GCps proliferation postnatally, and suggests that additional genetic or perhaps non-genetic factors are responsible for causing the pronounced vermis a/hypoplasia characteristic of Dandy–Walker malformation, perhaps by interacting with *Zic1* (Blank et al., 2011).

The analysis of *Foxc1* (forkhead box C1) mouse mutants after the identification of *FOXC1* as a candidate gene for Dandy–Walker malformation in humans, has revealed an novel mechanism whereby cerebellar vermis hypoplasia could arise. Mouse*Foxc1* hypomorphs exhibit vermis hypoplasia confirming that reduced FOXC1 function is responsible for Dandy–Walker malformation in humans. Intriguingly, *Foxc1* is not expressed in the developing cerebellum, but in mesenchymal tissue of the posterior fossa covering the cerebellar anlage from about E11.5. The IsO, roof plate, and RL initially develop normally in these mutants, but *Atoh1* expression in GCps in the medial EGL was lost by E14.5 of development. As *Atoh1* is required for GCp proliferation (Flora et al., 2009), these cells fail to expand resulting in the absence of an EGL in the medial cerebellum by birth. The exact mechanisms whereby FOXC1 in the cranial mesenchyme controls *Atoh1* expression in GCps are not known, but Aldinger et al. (2009) showed that the expression of BMP and TGFβ family genes are reduced in these mutant embryos. As BMP signaling is required for normal *Atoh1* expression and GCp expansion, this observation provides a likely explanation for vermis hypoplasia in patients with *FOXC1* mutations (Tong and Kwan, 2013). These findings highlight the important contribution of signaling interactions between progenitor zones and non-progenitor tissues like the cranial mesenchyme and roof plate in cerebellar development.

In conclusion, studies so far appear to primarily link Dandy–Walker Malformations to defects in GCp expansion. However, with the exception of *Foxc1*, the reasons for the disproportionate effect on vermis progenitors are not understood. It is interesting to note that both *Zic1* and *Zic4* are also expressed in the mesenchyme overlying the cerebellar anlage; perhaps these genes have an additional function in this tissue that might explain the vermis hypoplasia (Blank et al., 2011).

Pontocerebellar hypoplasia

Pontocerebellar hypoplasia is characterized by hypoplasia of the brainstem and cerebellum by birth; a condition to usually deteriorates further suggesting that some pontocerebellar hypoplasias can be classified as a neurodegenerative condition. Clinical signs include severe motor and developmental delays, respiratory deficiency and early postnatal lethality (Barth, 1993). The *CHMP1A* (charged multivesicular body protein 1a) gene that encodes chromatin modifying protein 1A (CHMP1A), has been identified as a candidate gene for pontocerebellar hypoplasia. Patient-derived lymphoblastoid cell lines showed reduced proliferation and increased expression of the cell cycle inhibitor INK4A (cyclin-dependent kinase inhibitor 2A, p16Ink4a), a target of the Polycomb group member BMI1 (B lymphoma Mo-MLV insertion region 1 homolog). This change in expression correlated with reduced BMI1 recruitment to an *Ink4a* regulatory region, suggesting that CHMP1A may regulate Polycomb recruitment

to gene loci, thereby phenocopying the cerebellar phenotype in *Bmi1−/−* mutants and (Mochida et al., 2012). To what extent the loss of BMI1 or other Polycomb components affects the development of pontine structures have not been established.

With the rapid advance in genomic science many genetic changes have now been identified to be associated with human pontocerebellar hypoplasia. These include intriguing candidates like *CASK* (calcium/calmodulin-dependent serine protein kinase), *EXOSC3* (exosome component 3), *RARS2* (arginyl-tRNA synthetase 2), *TSEN54* (tRNA-splicing endonuclease subunit 54), *TSEN2* (tRNA-splicing endonuclease subunit 2), and *TSEN34* (tRNA-splicing endonuclease subunit 34; Edvardson et al., 2007; Najm et al., 2008; Graham et al., 2010; Wan et al., 2012). The latter genetic associations suggest pathogenic mechanism related to defects in fundamental processes like protein synthesis and RNA splicing, but the mechanisms are likely to be varied and largely remain unexplored.

COGNITIVE CONSEQUENCES OF CEREBELLAR DEFECTS

In addition to well-established effects on motor coordination, the consequences of cerebellar defects on non-motor behaviors have received increasing attention over the last 20 years (Strick et al., 2009). Cerebellar lesions like those acquired after tumor resection have been linked to a number of non-motor behavioral disturbances. Schmahmann and Sherman (1998) coined the term "cerebellar cognitive affective syndrome" to describe the range of behavioral alterations associated with cerebellar lesions. These include alterations in (i) executive functions like planning and abstract reasoning, (ii) spatial recognition, (iii) language difficulties especially with grammar and controlling pitch and timing, and (iv) changes in measures of personality like emotions and social behavior (Schmahmann and Sherman, 1998; Tavano et al., 2007). Attempts to conceptualize this constellation of phenotypes have focused on the capacity of the cerebellum to accurately measure time intervals (Ivry et al., 1988; Ackermann et al., 1998; Schmahmann, 1998). The demonstrable ability of the cerebellum to generate internal models during learning (Imamizu et al., 2000) might thus be applied to generating internal models of cognitive processes via a series of re-entrant connections with non-motor cortical areas (Schmahmann and Pandya, 1991).

The anatomical basis for the influence of the cerebellum on cortical activity is the ascending projection from the dentate nucleus: a deep cerebellar nuclear formation that appears to be exclusive to mammals (Nieuwenhuys et al., 1998). The output of the dentate nucleus is modulated by Purkinje cells in the large neo-cerebellar hemispheres. In these regions the relatively coarse correspondence of cerebellar topography with particular body regions breaks down and is, at best, highly fragmented (Manni and Petrosini, 2004). In recent years, the advent of new trans-synaptic labeling techniques has allowed these cortical connections in primates, which were identified by traditional neuronal tracers (Schmahmann and Pandya, 1989), to be mapped with greatly improved precision. This has revealed that both primary motor cortex and dorsolateral prefrontal cortex are connected in "closed" loops with the cerebellum. In other words, Purkinje cells that modulate these cortical areas via the dentate nucleus, receive a reciprocal cortical input from the same regions via the pons (Kelly and Strick, 2003; **Figure 2C**). Furthermore, and contrary to prevailing theories (Glickstein, 2000), there is little anatomical cross-talk between these distinct loops. These observations sketch out a system of cerebello-cortical connectivity where substantial areas of the cerebellum are anatomically allocated to distinct cognitive processes. It is perhaps therefore unsurprising that the ventral portion of the dentate nucleus, which has been proposed to specifically mediate "cognitive" connections (Strick et al., 2009) is substantially greater in humans than other primates (Matano, 2001).

THE CEREBELLUM AND AUTISTIC SPECTRUM DISORDER

The increasing evidence for a cognitive role for the cerebellum has correlated with a number of studies that have linked forms of cerebellar hypoplasia with ASD (Courchesne et al., 1988, 1994; Bauman, 1991; Courchesne, 1997; Palmen et al., 2004; Dicicco-Bloom et al., 2006; Schmahmann, 2010; Fatemi et al., 2012). While firmly located within cortical processes (Mundy, 2003), these studies have prompted the hypothesis that ASD, might be mediated in part by interactions between cerebellum and cortex. By analogy, ASD represents a deficit in the cognitive equivalent of the modulatory processes by which the cerebellum has long been know to fine-tune motor skills and learning: a so-called "dysmetria of thought" (Schmahmann, 2010).

While this area of theory is necessarily in its early stages of development, it defines a new frontier for cerebellar research that has been probed both in human studies and experimentally in mice. Various groups have identified cerebellar vermis hypoplasia in patients with syndromic forms of autism (Becker et al., 2001; Sanlaville and Verloes, 2007; Aldinger et al., 2013). However, the precise link between cerebellar development and ASD are unclear. Studies of large-scale cerebellar pathology have revealed conflicting data with respect to the tempo of brain growth in patients and control groups (reviewed by Palmen et al., 2004). By contrast, at a cellular level, more substantive trends have emerged in terms of Purkinje cell loss (Ritvo et al., 1986; Kemper and Bauman, 1993) or reduction in cell size (Fatemi et al., 2002). Furthermore, some studies have shown an intriguing specificity in the location of Purkinje cell loss (Courchesne et al., 1988) with respect to areas activated during cognitive processes (Stoodley and Schmahmann, 2009). A small number of studies also examined specific alterations in cerebellar nucleus connections as assessed by diffusion tensor imaging (Brito et al., 2009; Sivaswamy et al., 2010) and histopathology (Bauman and Kemper, 1994). In particular, the superior (containing cerebellar output to thalamus) and middle (input from pons) cerebellar peduncles, which underlie cortical cerebellar loops, appear specifically affected.

From these findings it is apparent that ASD is accompanied by pathology in different cell types reflected by different developmental origins within different time windows. This fragmented pattern of pathology might reflect multiple different developmental aetiologies for a syndrome that is represented by a broad range of severity of disability. Alternatively, these observations may reflect coordinated patterns of trans-synaptic degeneration stemming from a single locus. The flipside to this perspective is retrograde neurodegeneration might predict patterns of cell loss that help establish the timing for developmental causes of ASD. As

Bauman and Kemper (2005) point out, the lack of retrograde loss of olivary neurons in ASD patients with reduced numbers of Purkinje cells suggests that cell death must have occurred prior to 28–30 weeks gestation (Bauman and Kemper, 2005).

Despite the potential complexities of cerebellar origins of ASD, a recent analysis of an ASD-like condition in a tuberous sclerosis complex 1 (*Tsc1*) mutant mouse is a particularly significant step in linking cerebellar defects to ASD (Tsai et al., 2012). Firstly, it establishes the parameters of a reference mouse model for human ASD, which is exhibited by many Tuberous Sclerosis patients. Secondly, Sahin and colleagues generated a mutant where gene deletion was restricted to the cerebellum and yet still recapitulated ASD-like behavior. This provides compelling evidence that developmental defects restricted to the cerebellum can result in a number of behaviors typical of autism. By deleting *Tsc1* only from cerebellar Purkinje cells during postnatal development, Tsai et al. (2012) could show that a reduction in Purkinje cells and increased Purkinje cell spine density were associated with altered behaviors typically associated with ASDs. The core behavioral features assessed during ASD diagnosis in humans include deficits in social reciprocity, communication, language delay, repetitive behaviors, and an insistence on sameness. Some of these behaviors can be modeled and was examined in these Purkinje cell-specific *Tsc1* mutant mice. These mice scored low on sociability and social novelty tests when presented with the opportunity to interact with a new mouse. Although communication and language are not easily assessed in mice, pups will attempt to attract the mother's attention by ultrasonic vocalizations when separated from her. *Tsc1* mutant pups showed an increase in these vocalizations, suggesting that the lack of *Tsc1* in the cerebellum can affect this process, although the relevance of this observation to ASDs is not clear. Finally, *Tsc1*-deficient mice exhibited excessive grooming, an indication of repetitive behaviors and demonstrated cognitive inflexibility in a reversal learning paradigm, perhaps indicative of an insistence of sameness (Tsai et al., 2012). This landmark study provides direct evidence that subtle disruptions in cerebellar architecture can have pronounced effects on behaviors typically associated with cortical defects. The promise of such mouse models lies not only in understanding the genetic basis of ASD but also the anatomical questions of where in the anatomical pathways of cortico-cerebellar connectivity gene deletions impose circuit-wide pathology.

FUTURE DIRECTIONS

The identification of the genetic defects responsible for specific anatomical abnormalities in the cerebellum is likely to continue at an unrivalled speed owing largely to the revolution in next generation sequencing. However, it should be remembered that knowledge of the genetic basis of disease is only the first step in understanding the condition. The next big challenge is to unravel the developmental and molecular mechanisms by which genetic changes manifest in a particular disease phenotype. Studies in model systems like the mouse have been essential in this quest and will no doubt continue to remain so. Without the fundamental knowledge gained from these experimental studies, translation of genetic findings to preventative and curative strategies will not be possible.

Unraveling the salient features and functional importance of cerebellar connectivity with cortical regions implicated in psychiatric conditions like autism, ADHD and schizophrenia, is of the utmost importance of we want to understand the involvement of the cerebellum in these conditions (Whitty et al., 2009; O'Halloran et al., 2012). Multi-disciplinary teams that combine the expertise of clinicians, radiologists, human geneticists, developmental and molecular biologists, and experts in rodent behavior are most likely to succeed in providing a more complete understanding of how genes and mechanisms that control cerebellar development relate to cerebellar disease and function.

Significant differences between the neuroanatomy of rodents and primates may emerge as barriers to understanding cognitive deficits in the mouse, despite all its advantages as an experimental and genetic model. Nevertheless, we highlight here that recent insights gained in the Tsc1 mutant suggest that the substrates for cortico-cerebellar modulation of behavior are present in the rodent. While this field is relatively undeveloped, the promise for understanding the full range of cerebellum function through exploration of these systems in mouse presents a research avenue of enormous promise.

Finally, this article almost exclusively focuses on cerebellar hypoplasia. Many other congenital cerebellar malformations have been described, including a range of conditions that appear to be associated with malformation of the posterior fossa and cerebellar overgrowth. For example, rasopathies like Costello syndrome are characterized by Chiari type I malformation and cerebellar tonsillar herniation (Gripp et al., 2010). One might like to speculate that abnormal signaling between the cranial mesenchyme and the cerebellar anlage, as those implicated in the aetiology of Dandy–Walker malformation could be involved in a subset of these conditions. However, the underlying mechanisms responsible for cerebellar defects in these conditions remain largely enigmatic. Epigenetic and environmental influences are also likely to contribute substantially to cerebellar disease burden and psychiatric diseases linked to cerebellar dysfunction. Investigative studies in the laboratory, aimed at unraveling the potential mechanisms whereby alterations that affect development through means other than changes in the coding sequences of genes cause disease are just beginning and represent an important piece of the puzzle (Pidsley et al., 2012; James et al., 2013).

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Cellular and molecular basis of cerebellar development

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Historically, the molecular and cellular mechanisms of cerebellar development were investigated through structural descriptions and studying spontaneous mutations in animal models and humans. Advances in experimental embryology, genetic engineering, and neuroimaging techniques render today the possibility to approach the analysis of molecular mechanisms underlying histogenesis and morphogenesis of the cerebellum by experimental designs. Several genes and molecules were identified to be involved in the cerebellar plate regionalization, specification, and differentiation of cerebellar neurons, as well as the establishment of cellular migratory routes and the subsequent neuronal connectivity. Indeed, pattern formation of the cerebellum requires the adequate orchestration of both key morphogenetic signals, arising from distinct brain regions, and local expression of specific transcription factors. Thus, the present review wants to revisit and discuss these morphogenetic and molecular mechanisms taking place during cerebellar development in order to understand causal processes regulating cerebellar cytoarchitecture, its highly topographically ordered circuitry and its role in brain function.

Keywords: rostral hindbrain, caudal mesencephalon, cerebellum, isthmus, isthmic organizer, isthmic constriction, Fgf8, morphogenesis

INTRODUCTION

The vertebrate brain is a remarkably complex anatomical structure that contains diverse subdivisions and neuronal subtypes with specific, sometimes prodigal, synaptic connections that contribute to the complexity of its function. During development the primordial brain (the neural tube) has to be progressively regionalized. A precise spatial and temporal arrangement of gene expression regulates intercellular and intracellular signals driving a proper molecular patterning that is required for this regionalization. Pioneering genoarchitectural studies and fate mapping experiments established correlations on how morphogens, transcription factors, and other signaling molecules modulate the specification of neuroepithelial territories, to generate the structural complexity and cellular diversity that characterizes the brain (revised in Puelles and Rubenstein, 2003; Martínez et al., 2012; Puelles and Ferran, 2012). Thus, the combination of molecular genetics (gene expression maps) and modern neuroanatomy (based on histochemistry and highly sensitive neuroimaging) have led to an increased interest in describing the neurodevelopmental mechanisms underlying structural disorders and intellectual discapacities that we currently observe in congenital anomalies of the human brain.

Among the classical systems used to study the structure and function of the central nervous system the cerebellum has steadily gained popularity and has become one of the most experimentally tractable systems in the brain. Much of our knowledge about structure, function, and development of the mouse cerebellum was achieved by studying spontaneous mutations (Sotelo, 2004), but also by using sophisticated genetic tools allowing a more precise and mechanistic level of analysis (Joyner and Sudarov, 2012; Tvrdik and Capecchi, 2012).

This review focuses on the basic developmental biology of the cerebellum starting from morphological features in order to distinguish the origin and specification of the cerebellar neuroepithelial anlage, as well as to describe the molecular mechanisms implicated in the development of its architectural morphology, stereotyped cellular differentiation, and neuronal distribution. Finally, we summarize relevant works in correlations with those findings in developmental cerebellar disorders of the human cerebellum (Barkovich, 2012).

THE TOPOGRAPHY AND TOPOLOGY OF THE CEREBELLAR ANLAGE

The CNS arises from an apparent homogenous sheet of epithelial cells, the neural plate, induced during gastrulation by the dorsal lip of the blastopore in amphibians (Spemann and Mangold, 1924) or by the Hensen's node in amniotes. During the process of neural induction the neural plate pursues morphological differentiation, its edges thicken and roll up, to close dorsally in order to form the neural tube. The most anterior portion of the neural tube is undergoing drastic changes during early development generating, by differential proliferation, the three primary brain vesicles: the forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon); caudal neural tube remains with a cylindrical shape and generates the spinal cord (Martínez and Puelles, 2000). The discovery that putative regulatory genes are expressed in regionally restricted patterns in the developing neural tube has provided new tools for defining histogenic domains and their boundaries at higher resolution. In the rhombencephalon, the segments are termed rhombomeres (r) that from anterior to posterior are known as r0 (the isthmus) and r1–r7, followed by the pseudorhombomeres

r8–r11 (Marín and Puelles, 1995; Cambronero and Puelles, 2000; **Figure 1A**). The mature cerebellum is composed of two cerebellar hemispheres and the vermis located between these hemispheres. Embryonically the cerebellar hemispheres and the vermis originate from the first two rhombomeres. The vermis is part of the alar r0 and roof plates of r0 and r1, whereas the hemispheres belong to the alar r1 (**Figures 1A**, **3A**,**B**). Already in 1890, Wilhelm His proposed the alar neuroepithelium of the anterior rhombencephalon (or metencephalon) as the origin of the cerebellum. He postulated that from these paired plates, the cerebellum evolves as a bilateral organ, which would subsequently fuse at the dorsal roof midline in a rostral-to-caudal direction, to form a uniform primordium.

The topographical boundary between the mesencephalon and rhombencephalon is the isthmic constriction or simply the isthmus (Isth; **Figure 1B′**). This was initially interpreted to bridge the midbrain-hindbrain boundary (Alvarado-Mallart, 1993), but is now thought to co-localize with the prospective isthmic territory (r0), as defined early on by the expression of the well-known secreted molecule fibroblast growth factor 8 (*Fgf8*; Crossley et al., 1996). Homotopic and isochronic quail-chick grafting experiments performed in the late 1980's and the 1990's consistently showed that the caudal part of the early midbrain vesicle had a peculiar morphogenesis and generated the rostral and medial part of the prospective cerebellum (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990; Alvarez-Otero et al., 1993; Marin and Puelles, 1994; Hidalgo-Sánchez et al., 2005). Therefore, the anterior vermal part of the presumptive cerebellum, instead to result from fusion of lateral cerebellar plates (His, 1889), originated from the caudal and alar portion of the mesencephalic vesicle. Hence, Puelles and collaborators argued that the early mid-hindbrain constriction observed by Vaage (1969) was not

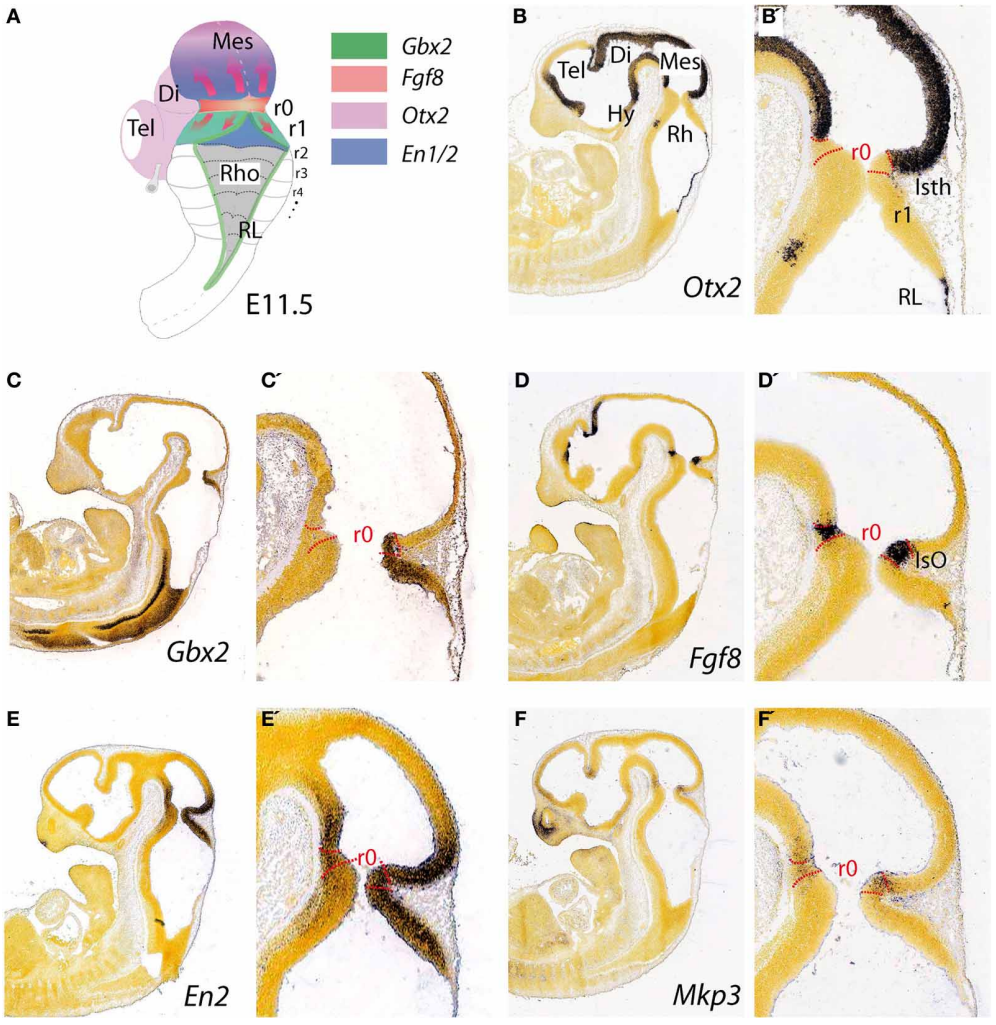


FIGURE 1 | Topographical location and main molecular characterization of the mid-hindbrain boundary at E11.5. (A) A dorsal view of an E11.5 mouse embryo illustrating the isthmic constriction (isth) located between the mesencephalon and rhombomere 1 (r1). Moreover, rhombomeres, r0 and r1, which give rise to the cerebellum are highlighted in **(A)**. The different color codes depict the expression pattern of the most important genes related to the morphogenetic activity and the capacity of the IsO (We only consider *Fgf8*-positive territory at this constriction as IsO; see also Martínez, 2001). Expression patterns of genes that are illustrated in **(A)** and their boundaries are also shown by *in situ* hybridization (ISH) at E11.5 for *Otx2* **(B,B′)**, *Gbx2* **(C,C′),** *Fgf8* **(D,D′)** *En2* **(E,E′),** and *Mkp3* **(F,F′)**. Panels (**B–F′**) were taken from Allen Institute for Brain Science public resources (http://www.brain-map.org/).

a fixed non-proliferative neuroepithelial structure, as proper interneuromeric landmarks are, but it was a wave-like transient conformation of the local proliferating neuroepithelium. In avian embryos gene expression pattern analysis has proved that the relative position of the actual midbrain-hindbrain boundary, is located at the edge of *Otx2* and *Gbx2* expression domains (initially inside mesencephalic vesicle) and moves caudally after stage HH15, to coincide with the pre-existent midbrain hindbrain constriction at around HH20–21 (Martinez and Alvarado-Mallart, 1989; Alvarez-Otero et al., 1993; Hidalgo-Sánchez et al., 2005; **Figures 1B**,**C**).

The homeodomain transcription factors of Engrailed family *En1* and *En2* (**Figures 1E**,**E′**) are expressed early on in cerebellar and mesencephalic primordial neuroepithelium and both are involved in the formation of the mesencephalic tectum and cerebellum (**Figure 1A**). Thus, mouse *En1* mutants lack most of the tectum and cerebellum and die at birth, whereas *En2* mutants are viable with a smaller cerebellum and foliation defects (Joyner et al., 1991; Hanks et al., 1995). Experimental studies indicate that the severeness of *En1* and *En2* phenotypes differs due to a relatively early onset of *En1* expression compared to the onset of *En2* expression, rather than differences in protein function (Joyner et al., 1991; Millen et al., 1995). Studies on conditional mutant alleles of *En1* and/or *En2* demonstrated that *En1* is required for cerebellar development only before embryonic day 9, but plays a substantial role in forming the tectum. In fact, *En2* was found to be more potent than *En1* in cerebellar development (Sgaier et al., 2007). In addition these authors proved that there is an *En1/2* dose-dependent genetic subdivision of the tectum into its two functional alar subdivisions (anterior and posterior colliculi) and of the medio-lateral cerebellum into four regions that have distinct molecular coding and represent functional domains.

THE MOLECULAR SPECIFICATION OF THE CEREBELLAR ANLAGE: THE ISTHMIC ORGANIZER

Distinct neural and glial identities are acquired by neuroepithelial progenitor cells through progressive restriction of histogenetic potential under the influence of local environmental signals. Evidence for morphogenetic regulatory processes at specific locations of the developing neural primordium has led to the concept of secondary organizers, which regulate the identity and regional polarity of neighboring neuroepithelial regions (Ruiz i Altaba, 1998; for review see Echevarría et al., 2003). Thus, these organizers, secondary to those that operate throughout the embryo during gastrulation, usually develop within the previously broadly regionalized neuroectoderm at given genetic boundaries (frequently where cells expressing different transcription factors are juxtaposed). Their subsequent activity refines local neural identities along the AP or DV axes and regionalizing the anterior neural plate and neural tube (Meinhardt, 1983; Figdor and Stern, 1993; Rubenstein and Puelles, 1994; Shimamura et al., 1995; Wassef and Joyner, 1997; Rubenstein et al., 1998; Joyner et al., 2000).

Three regions in the neural plate and tube have been identified as putative secondary organizers: the anterior neural ridge (ANR) at the anterior end of the neural plate, the zona limitans intrathalamica (ZLI) in the diencephalon, and the isthmic organizer (IsO) at the mid-hindbrain boundary (Vieira et al., 2010). Therefore, the isthmic constriction contains the IsO (**Figures 1D**,**D′**), which has been extensively studied during the last decade (Martinez and Alvarado-Mallart, 1989; for review see Martínez, 2001; Wurst and Bally-Cuif, 2001; Echevarría et al., 2003; Aroca and Puelles, 2005; Hidalgo-Sánchez et al., 2005; Nakamura et al., 2005; Partanen, 2007). It is involved in maintaining the mid-hindbrain boundary and providing structural polarity to the adjoining regions in order to orchestrate the complex cellular diversity of the mesencephalon (rostrally) and the cerebellum (caudally; Itasaki and Nakamura, 1992; Martínez, 2001; Rhinn and Brand, 2001; Crespo-Enriquez et al., 2012).

The earliest molecular event for the IsO specification is the differential expression in the neural plate of *Otx2* in the rostral epithelium and a *Gbx2* in the posterior domain (Wassarman et al., 1997; Shamim and Mason, 1998; Broccoli et al., 1999; Katahira et al., 2000; **Figures 1B**,**C′**, **4**). In the avian embryo at HH8 an *Otx2* and *Gbx2* negative neuroepithelial gap separate these domains, but at stages HH9–10 they come to overlap across the prospective mid-hindbrain boundary (Garda et al., 2001). Some of the key experiments, revealing the molecular nature and regulation of the signals for the specification of the IsO, were performed already 18 years ago. A member of the fibroblast growth factor (FGF) family, *Fgf8*, was found to be highly expressed in the most anterior hindbrain (Heikinheimo et al., 1994; Crossley and Martin, 1995; **Figure 1D**). Furthermore, beads-containing FGF8 protein were found to effectively mimic the activity of the IsO tissue when transplanted either into the diencephalon or posterior hindbrain (Crossley et al., 1996; Martinez et al., 1999). Since these experiments, members of the FGF8 subfamily (Itoh and Ornitz, 2004) have been shown to be also morphogenetic signals that regulate structural aspects of midbrain, isthmus (Isth), and r1 development (**Figures 1A**,**D**,**D′**).

Fgf8 expression is first activated at HH9+ in birds and at E8.5 in mice at the interface of *Otx2* and *Gbx2* positive neuroepithelial cells. WNT1 and EN2 proteins are already expressed at this stage across the incipient boundary, with a maximum expression level at the *Fgf8* positive domain, showing decreasing gradients oriented either rostrally toward mesencephalic epithelium or caudally toward rhombencephalic epithelium, respectively. The co-expression of *Otx2* and *Gbx2* in the IsO territory essentially disappears by HH11–12 and both domains become thereafter mutually excluded and complementary (Millet et al., 1999; Garda et al., 2001; Liu and Joyner, 2001). The caudal limit of *Otx2* expression and the rostral limit of *Gbx2* therefore mark the mid-hindbrain molecular boundary (MHB; Millet et al., 1996; Hidalgo-Sánchez et al., 1999; Martínez, 2001). Secondarily, *Lmx1b* and *Wnt1* are co-expressed in a thin band confined to the caudal most *Otx2* expression domain, abutting the *Fgf8* domain at the rostral most edge of the hindbrain. *Lmx1b* activates *Wnt1* in a cell-autonomous manner and represses *Fgf8* in a non-autonomous way, thus contributing to maintain the rostral limit of *Fgf8* at the MHB (Matsunaga et al., 2002) and thus being essential for the initial steps of mid-hindbrain development (Guo et al., 2007). Note that although early *Fgf8* expression appears in the territory co-expressing *Otx2/Gbx2*, double deletion of these two transcription factors in the mouse does not affect the activation of *Fgf8* expression (Li and Joyner, 2001; Martinez-Barbera

et al., 2001). Other genes expressed at very early stages across the prospective MHB, such as *Pax2* (Rowitch and McMahon, 1995; Joyner, 1996; Hidalgo-Sánchez et al., 2005) and *Iroquas* (*Irxs*) seem required for the expression of *Otx2*, *Gbx2*, and *Fgf8* and the proper formation of the mesencephalic and rhombencephalic vesicles (Vieira et al., 2010). Recently it was proposed that *Gbx2* and *Fgf8* are sequentially required for formation of the mid-hindbrain boundary, playing a crucial role in maintaining here a boundary of cell lineage by restricting cell movement (Sunmonu et al., 2011; **Figure 4**).

Moreover, FGF8 signal may act at the IsO in concert with other signaling molecules, such as WNT1, Sonic Hedgehog (SHH) and transforming growth factor (TGF)-β family members (Danielian and McMahon, 1996; Matsunaga et al., 2002; Vogel-Höpker and Rohrer, 2002; Castelo-Branco et al., 2003; Farkas et al., 2003; Blaess et al., 2006). The morphogenetic activity of the IsO is then a consequence of a specific temporo-spatial expression of molecular signals, which regulate the specification and structural development of mesencephalic and cerebellar neuroepithelial territories. Alterations of *Fgf8* and *Gbx2* gene expression lead to massive disruption of the mid-hindbrain neural territory by gene patterning dysregulation (Wassarman et al., 1997). A decreasing gradient of FGF8 protein concentration in the alar plate of the isthmus and r1 is fundamental for cell survival and the differential development of cerebellar regions (Chi et al., 2003; Nakamura et al., 2005; Basson et al., 2008). In the basal plate, FGF8 gradient is crucial for cell survival and, together with SHH, essential for the development of caudal serotonergic and rostral dopaminergic fates of progenitor cells, as well as the localization and development of other basal derivatives, such as noradrenergic cells in the locus coeruleus (in the rhombencephalon) and the red nucleus (in the mesencephalic tegmentum; Wurst and Bally-Cuif, 2001; Chi et al., 2003; Puelles and Rubenstein, 2003; Prakash and Wurst, 2006; Prakash et al., 2006). On the other hand, mesencephalic and diencephalic epithelia are also receptive to FGF8 (Martinez et al., 1991, 1999; Crossley et al., 1996; Crespo-Enriquez et al., 2012), which possibly regulates gene expression and neuroepithelial polarity in the alar plate of these territories (Vieira et al., 2006; Crespo-Enriquez et al., 2012).

Finally, the proposed mechanism by which FGF8 signaling spreads over a field of target cells, at least in zebrafish, is established and maintained by two essential factors: firstly, free diffusion of single FGF8 molecules away from the secretion source through the extracellular space and secondly, an absorptive function of the receiving cells regulated by receptor-mediated endocytosis (Yu et al., 2009; Nowak et al., 2011; Müller et al., 2013). Several studies have disclosed the position preferences of neuroepithelial cells to FGF8 planar signal activity. The differential orientation and polarity of the FGF8 signal seems to be directly dependent on the spatial position of mouse Fgf8-related secondary organizers and on the activity of the negative modulators, *Mkp3* (**Figures 1F**,**F′**; Echevarria et al., 2005a,b), *Sef* (Fürthauer et al., 2002; Tsang et al., 2002), and *Sprouty1/2* (Spry1/2; Minowada et al., 1999; Echevarria et al., 2005b; **Figure 4**). Relevant published findings in chick embryos claimed that FGF8b may also translocate into the nucleus, and this nuclear FGF8b could function as a transcriptional regulator to induce *Spry2* in the isthmus independently of ERK phosphorylation (Suzuki et al., 2012). Similar findings in mouse found maintenance of *Spry2* expression pattern along the Isthmic region in temporally absence of FGF8 in the extracellular compartment, as well as ERK phosphorylation (Crespo-Enriquez et al., 2012). The latter findings reaffirm the existence of positional information encoded by the FGF8 signal through planar transcellular corridors in neuroepithelial cells along the vertebrate neural tube.

HISTOGENESIS AND CELLULAR IDENTITY OF THE CEREBELLAR ANLAGE

The cerebellum is indeed a unique brain structure dependent of FGF8 signal and *Gbx2* homeobox expression. The medial part is know as the vermis and develops from the isthmic and r1 roof plates, while the lateral parts are know as the cerebellar hemispheres and develop from the cerebellar plates at the r1 alar region (**Figures 3A**,**B**; Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990; Sotelo, 2004; Zervas et al., 2005). The cerebellum is further divided into cortex, white matter, and cerebellar nuclei. The cortex occupies the entire surface and is greatly increased in extension by the characteristic lobulations and foliations, which from a midsagittal section have the appearance of a "tree" (**Figure 2E**). The cerebellar foliation is consequence of mechanical forces that induce fissure formation (see color-coded folia identification formation in **Figures 2C–E**). It has been proposed different causal mechanisms for cerebellar foliation: one possibility for folia formation in the cerebellum is because Purkinje cells (PCs) anchors the cortex to the underlying white matter via their axons at positions that define the base of fissures (Altman and Bayer, 1997). Alternatively, differential rates of granule cell precursor proliferation, with highest rates at the base of the fissures, have been suggested to underlie the postnatal growth of folia (Mareš et al., 1970). Recent studies have identified a reproducible series of cellular changes that the three major cerebellar cell types (PCs, granule cells, and Bergmann glia) undergo during initial formation of fissures demonstrating that the timing of these cellular changes governs folial shape (Sudarov and Joyner, 2007). The latter authors proposed a new model for cerebellar cortex folia specification whereby changes in the behavior of granule cell precursors drive the formation of "anchoring centers" at the base of each fissure consisting of PCs, granule cells, and Bergmann glia cells. Then folia outgrowth continues by a self-sustaining process involving the coordinated action of both granule cells and Bergmann glia. *En1/2* homeobox genes have been found to be crucial for the production of the distinct medial (vermal) and lateral (hemisphere) foliation patterns in mammalian cerebellum. Thus, these genes are proposed as a new class of genes that are fundamental for patterning cerebellum foliation throughout the medio-lateral axis acting late in development (Cheng et al., 2010; Orvis et al., 2012).

Another interesting aspect of the cerebellar cortex is its quite stereotyped cytoarchitecture. The neuronal subtypes are connected to each other in the same manner, building a cerebellar microcircuit (see below). However, and despite the well-known participation in coordinating proprioceptive-motor processing, the cerebellum is found to be involved in other very important

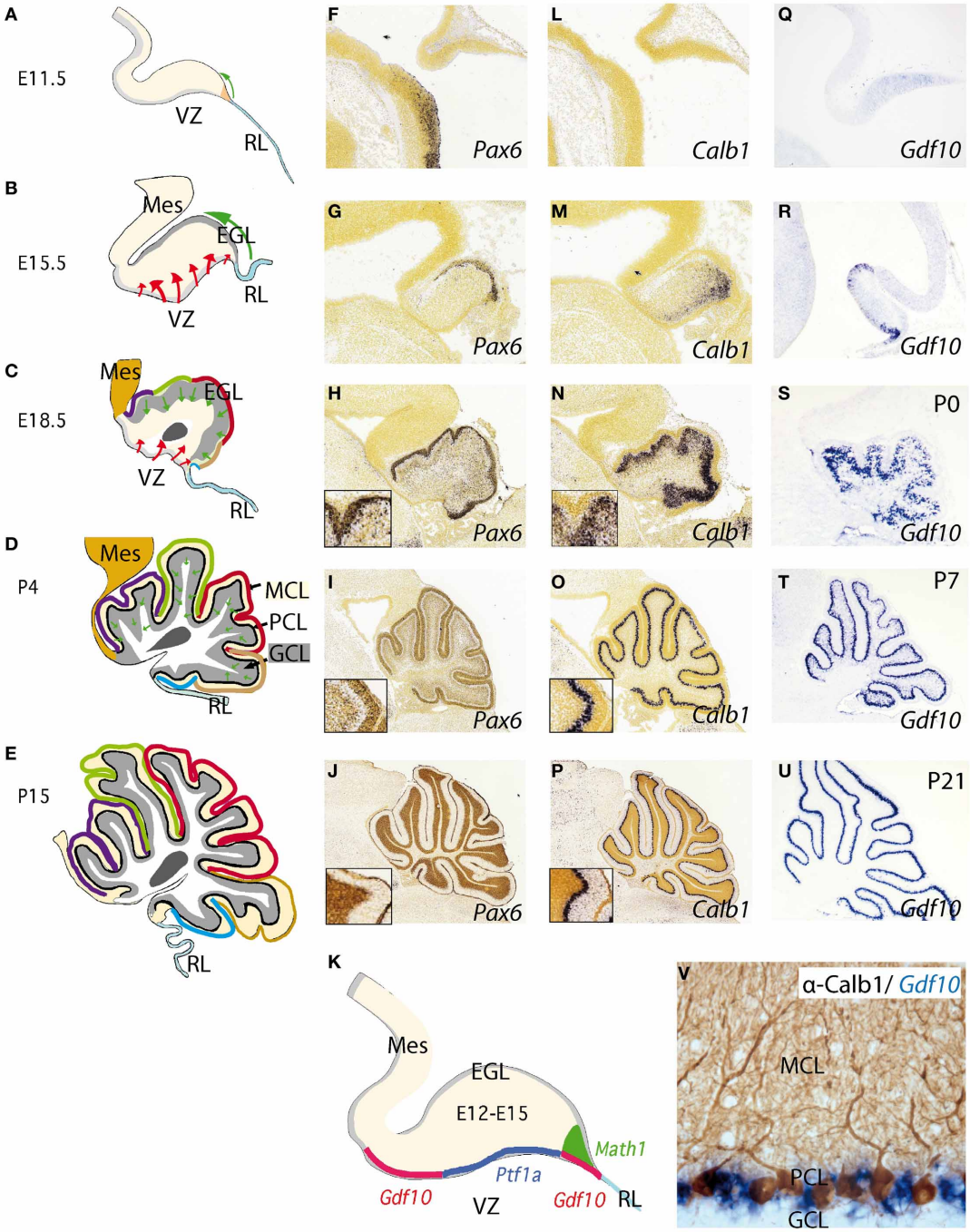


FIGURE 2 | Development of the cerebellum from the rhombencephalic alar plate from early stages to adulthood. (A–E) Cerebellar morphology/anatomy at different stages of development. The cerebellar folia development is showed from E11.5 onwards identifying the corresponding folia primordia bulges as color code lines from rostral to caudal (see also Sudarov and Joyner, 2007). Tangential migration from the rhombic lip (RL) is indicated as a green arrow corresponding to excitatory granule precursor cells that are specified by the expression of *Math1* **(K)**. These cells build up the external granule cell layer and from neonatal stages to P14, they descend radially forming the internal granule cell layer (GCL) located below Purkinje cell layer (PCL). The red arrows highlight the radial migration of cells originating from the ventricular zone (VZ). This is where Purkinje cells (PCs) and GABAergic cells of the cerebellum are born and specified by the early expression of *Ptf1a* **(K)**; see also Dastjerdi et al. (2012). **(F–U)** Represent *in situ* hybridizations (ISH) of corresponding markers for each cerebellar cell type. *Pax6* labeling granule cells **(F–J)**. From **(H)** to **(J)** the insert shows the position of *Pax6*-positive cells at the EGL and later on, in the GCL **(J)**. In the same manner (**L–P**) represent *Calbindin* (*Calb1*) showing the early location of PCs and their migration from the VZ to the final Purkinje cell monolayer, PCL (see also Sotelo, 2004). (**Q–U**) shows the expression pattern of *Gdf10* (a marker for Bergmann glial cells) by means of ISH during cerebellar development. **(V)** Here we show the expression of *Gdf10* in Bergmann glial cells in adult mice, together with Calbindin- positive Purkinje cells. **(K)** represents the molecular specification of the different cell types (excitatory vs. inhibitory) in the cerebellum including the two germinal centers ventricular zone (VZ) and rhombic lip (RL). Panels (**F–J**) and (**L,M**) were taken from Allen Institute for Brain Science public resources (http://www.brain-map.org/).

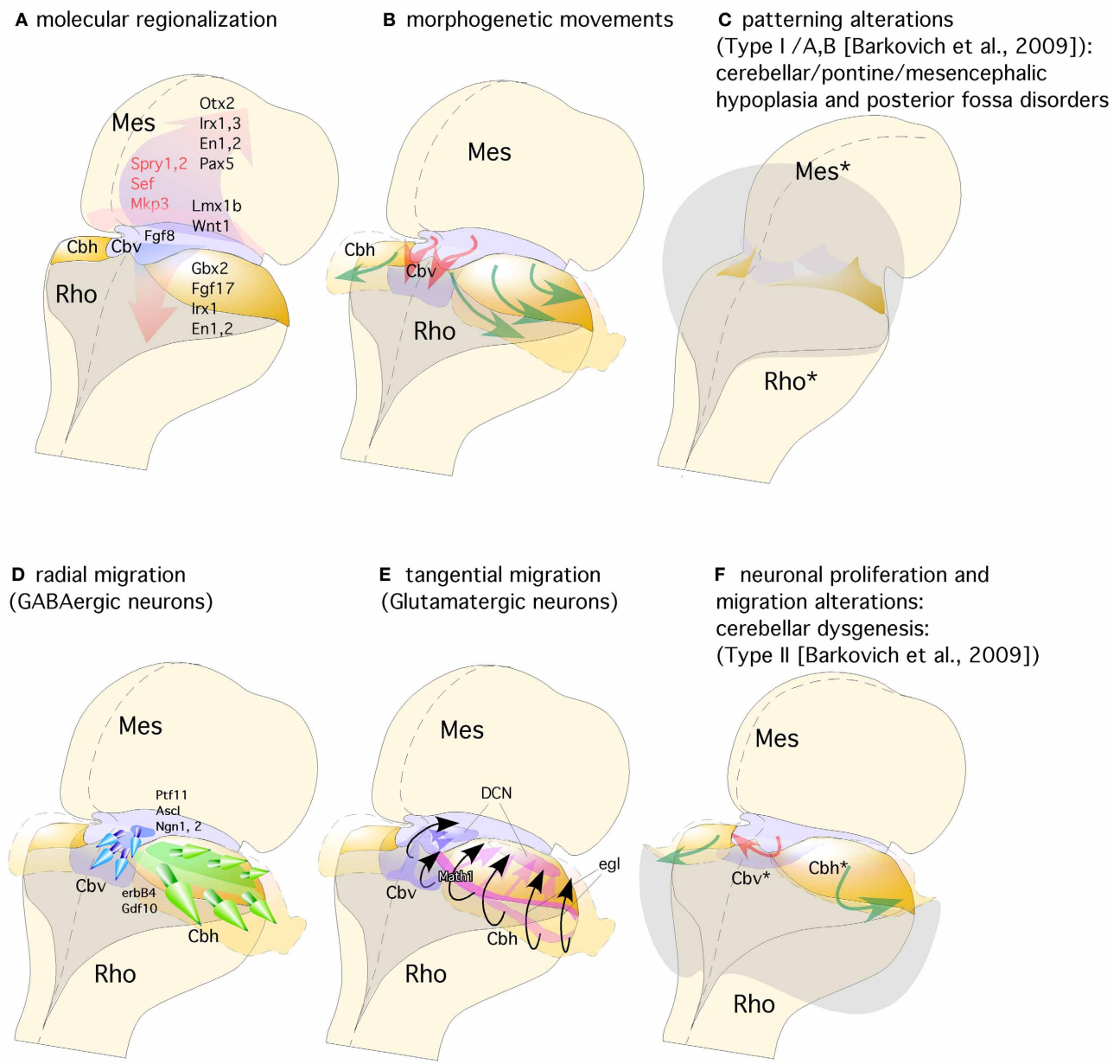
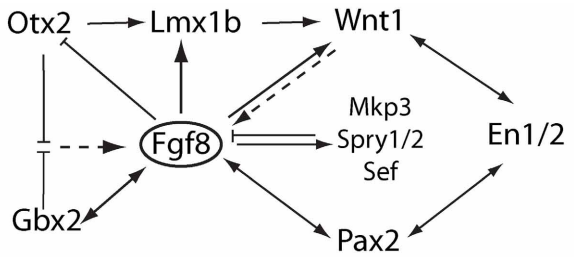


FIGURE 3 | Representation of a dorsal view of mid-hindbrain junction, where the isthmic segment is colored in light purple (which will generate also cerebellar vermis: Cbv) and the cerebellar plates in yellow (which will generate cerebellar hemispheres). (A) The isthmic organizer expressing *Fgf*8 (pink arrows) induces the expression of *Sprouty*, *Sef*, and *Mkp3* in this region; and is also required for other genes differentially expressed in the midbrain (Mes) or rhombencephalic (Rho) neuroepithelium. **(B)** The spatio-temporal expression of these genes regulates the normal morphogenesis and growth of the cerebellar vermis (Cbv; red arrows) and hemispheres (Cbh; green arrows). **(C)** Failure in proper isthmic organizer development (due to a lack of morphogenetic signaling or disruption of gene expression) can result in cerebellar (Rho*) and mesencephalic (Mes*) hypoplasia due to a strong increase of cell death with posterior fossa disorders and fourth ventricle dilatation (gray shadow). **(D)** Radial migration of GABAergic neurons in the cerebellar vermis (Cbv; blue arrows) and hemispheres (Cbh; Green arrows). **(E)** Rhombic lip specification is regulated by *Math1*. Tangential migration of glutamatergic neurons of the deep cerebellar nuclei (DCN) and granule cells (egl) are represented by pink and black arrows, respectively. **(F)** When normal development of cortical cerebellar cells is disrupted, the structural phenotype is classified as cerebellar dysgenesis (Cbv* and Cbh*), with enlargement of the fourth ventricle and reversion of cerebellar-choroidal junction (arrows).

higher functions such as cognition, emotion, and language processing (Zervas et al., 2005; Barkovich, 2012).

The adult cerebellar cortex is laminated into three layers. The molecular cell layer (MCL), rich in neuropil consisting mainly of parallel fibers, purkinje dendrites, and glial cell processes as well as neurons allocated at superficial and deep zones, such stellate and basket cells (**Figure 2D**). The PC layer (PCL) is composed of a monolayer of Calbindin positive PCs (**Figures 2D,L–P**),



H = homebox
TF= transcription factor
M= morphogen
EP= extracellular protein
E = intracellular enzyme
inh= inhibitor of FGF8 signalling

	Otx2	Gbx2	Wnt1	Lmx1b	En1	En2	Pax2	Fgf8	Mkp3	Spry1	Spry2	Sef
	H/TF	H/TF	M/EP	H/TF	H/TF	H/TF	H/TF	M/EP	E/Inh	E/Inh	E/Inh	E/Inh
Di	+++											
	+++											
Mes	+++					+					+	
	+++		+++	+++	++	++			+	++	++	++
Isth		+++		+	+++	+++	+++	+++	+++	+++	+++	+++
R_1		+++			++	++	++		++	++	++	++
R_2												
R_3												

FIGURE 4 | The upper scheme represents the functional interaction (induction/inhibition) of genes that, together with *Fgf8,* are involved in the molecular maintenance of isthmic region at E9.5. The table below summarizes the expression intensity and range of genes along the AP axis of the neural tube focusing on the isthmus. The color code depicts their mRNA expression range from the isthmus toward rostral or caudal regions.

candelabrum cells (Lainé and Axelrad, 1994), and Bergmann glia (see below; **Figure 2V**). The final and deepest layer is the so-called granule cell layer (GCL) and is the widest cerebellar layer, mainly composed of Pax6 positive granule cells (**Figures 2D**,**F–K**) as well as Golgi, Lugaro, and unipolar brush cells (Sotelo, 2004; Zervas et al., 2005). PCs are the only output of the cerebellar cortex, while inputs coming to the granular cells are transmitted to PCs via their axons, the parallel fibers. On the other hand, inhibitory interneurons such as stellate and basket cells innervate dendrites and soma of PCs respectively. Deeply, with respect to the cortex, the white matter is located in the center of the cerebellum. It extends to the ventricular surface of the 4th ventricle, accommodating the cerebellar nuclei (from medial to lateral: the fastigial, the interpositus, and the dentate nucleus). Axons coming from diverse cerebral origins enter the cerebellum through any of the cerebellar peduncles (superior, middle, and inferior). They project either directly or collaterally to the cerebellar nuclei and to the GCL in the cortex, and are named mossy fibers. Only those axons coming from the inferior olive are called climbing fibers because of their "climbing" features to synapses the PC dendrite arborization (Sotelo, 2004).

Neurochemically the cerebellar cortex contains two glutamatergic neuronal subtypes (granule and unipolar brush cells) and five GABAergic subtypes (Purkinje, Golgi, Lugaro, Stellate, and Basket cells). The deep cerebellar nuclei (DCN; **Figure 2C**), contain both GABAergic interneurons and glutamatergic projection neurons (Wang and Zoghbi, 2001; Hoshino, 2006; Leto et al., 2006; Carletti and Rossi, 2008). Fate-mapping studies of the developing cerebellum have uncovered when and where cells are born and which migratory routes they follow in order to reach their final position. Cerebellar neurons are generated from two major germinal centers: the external granule layer (EGL) and ventricular zone (VZ; Sotelo, 2004; Millen and Gleeson, 2008; **Figures 2B**,**C**). Over the past decades has been proven that granule cells are produced by early granule cell precursors located in the EGL that originate from the rhombic lip (RL; **Figures 1A**,**B**,**B′**, **2A–E**, **3D**,**E**), at the interface of the dorsal neural tube and the extended roof plate of the 4th ventricle (the choroid plexus; Chp; Wingate, 2001). Also, the glutamatergic DCN neurons and unipolar brush cells are derived from the RL (Fink et al., 2006; Carletti and Rossi, 2008). Therefore, all glutamatergic neurons in the cerebellum appear to originate from the RL. The anterior RL expresses *Math1* (also called *Atoh1*) as early as embryonic stage E9.5 in mice. *Math1* is induced by bone morphogenetic protein (BMP) from the roof plate, which itself is differentiating into the Chp (Basson et al., 2008; **Figures 2A–E**). *Math1* positive RL progenitor cells give rise to multiple glutamatergic cell derivatives in a time-dependent sequence. Progenitors of the rostral part of the RL migrate through over the cerebellar anlage and give rise to granule progenitors cells and DCN (**Figures 3D**,**E**). The caudal part of RL gives rise to multiple brain stem precerebellar nuclei, including the pontine nuclei and superior and inferior olive. Thus, *Math1* positive RL cells (**Figures 2K**, **3E**) generate cerebellar granule cells which mature in the EGL and later migrate inwards into the definitive granular cell layer in a anterior to posterior temporal gradient (green arrows in **Figures 2A–D**, **3D**,**E**; Sotelo, 2004). Unipolar brush cells are the last *Math1*-positive RL population migrating through the cerebellar white matter to their final GCL

locations (Millen et al., 1999; Bermingham et al., 2001; Wang et al., 2005; Millen and Gleeson, 2008). Yet, during the first 2 postnatal weeks in mice, granule cell precursors continue differentiating and migrating radially through MCL and PCL layers to form the final internal GCL leaving their bifurcated axons in the MCL (the parallel fibers; Hatten and Heintz, 2005).

The second germinal center, the VZ, has been thought to give rise to cerebellar GABAergic neurons (Altman and Bayer, 1997; Sotelo, 2004; Hoshino, 2006; Sudarov and Joyner, 2007; Carletti and Rossi, 2008; red arrows in **Figures 2B,C,K**). A recent genetic inducible fate-mapping study demonstrated that dorsal r1 first undergoes an orthogonal rotation such that the anterior posterior axis (A-P axis) of r1 at E9.5 becomes the medio-lateral axis (M-L axis) of dorsal r1 at E12.5 (Sgaier et al., 2005; **Figure 3B**). The M-L axis of ventricular-derived cells is then retained: PCs generated medially are located in the vermis while laterally generated PCs populate the hemispheres (Sgaier et al., 2005, 2007). The pancreas transcription factor 1 (*Ptf1a*), which encodes a bHLH transcription factor, is expressed at the VZ (**Figures 2K**, **3D**; Hoshino et al., 2005; Hoshino, 2006). The characterization of a novel mutant mouse, *Cerebelless*, which lacks the entire cerebellar cortex but still survives into adulthood, has clarified that *Pft1a* is required for generating all cerebellar GABAergic neurons including the PCs. Thus, *Math1* and *Ptf1a* participate in regionalizing the cerebellar neuroepithelium, and define two distinct territories, the VZ (*Ptf1a* positive) and the upper RL (*Math1* positive), which generate GABAergic and glutamatergic neurons, respectively (Hoshino et al., 2005; Pascual et al., 2007; **Figures 2K**, **3D**,**E**). The earliest markers for GABAergic PC progenitors express *Neph3*, *E-cadherin* (Mizuhara et al., 2010) as well as *Corl2* (Minaki et al., 2008) in the ventricular and subventricular zone while in post-mitotic PCs neurons other molecular determinants such as Calbindin, is expressed (**Figures 2L–P**; Sotelo, 2004; Muguruza and Sasai, 2012). Moreover, the expression domains of three pro-neural genes (*Ascl1*, *Neurog1*, and *Neurog2*) overlap with that of *Ptf1a* in the VZ (Zervas et al., 2005; Zordan et al., 2008; Dastjerdi et al., 2012; **Figure 3D**). A closer analysis of the role of *Ascl1* in cerebellar neurogenesis, established that *Ascl1* positive progenitors progressively delaminate out of the VZ to settle first in the prospective white matter, and then in the cerebellar cortex (Grimaldi et al., 2009). These authors demonstrated by gain of function experiments of *Ascl1* an increase of *Pax2* positive interneurons and *Olig2* positive oligodendrocyte precursors, while glutamatergic neurons, astrocytes, and Bergmann glial (BG) cells were not affected. On the other hand, the lack of *Ascl1* led to a dramatic reduction of *Pax2* and *Olig2* precursors. Interestingly, no change was found in PC development in any of the experiments mentioned above. Thus, the latter evidence suggests that *Ascl1* contributes to the generation of GABAergic interneurons and DCN as well as to PC development but not to their specification.

In addition to GABAergic neurons, progenitor cells located in the VZ of the fourth ventricle also give rise to BG cells. During development, the processes of BG provide structural support to the expanding cerebellar plate (see below). In addition radial Bergmann fibers act as essential guide rails for the migration of granule cells (Rakic, 1990) and contribute to the elaboration of PC dendrites (Yamada et al., 2000) and stabilize synaptic connections onto these neurons (Iino et al., 2001). Indirect evidence suggests that neuregulin, a member of the epidermal growth factor family, and its membrane receptor erbB4 are involved in the cerebellar induction of the radial glial scaffolds for granule cell migration (Rio et al., 1997). Migrating granule cells, as well as their EGL precursors (green arrows in **Figures 2A–D**), express neuregulin, whereas Bergmann fibers express erbB4 in the postnatal cerebellum. Moreover, activation of the receptor with soluble neuregulin mimics the effects of neuron–radial glial interactions in the induction of radial glial formation. In contrast, when the glial erbB4 receptors are inactivated by transfection with a dominant-negative form of erbB4, granule cells and soluble neuregulin fail to induce the radial glial phenotype *in vitro* (Rio et al., 1997).

BG cells, like interneurons and PCs, are born in the VZ of the fourth ventricle, where they express among other lineage-restricted markers growth and differentiation factor 10 (*Gdf10*; **Figures 2Q–V**; Alcaraz et al., 2006; Koirala and Corfas, 2010). In mice *Gdf10* was identified as a marker expressed in the PCL of the cerebellum (Zhao et al., 1999). Based on a publication by Zhao and colleagues, *Gdf10* is expressed in PCL but not in GCL. Finally, it was further demonstrated that *Gdf10* is expressed in BG cells (Koirala and Corfas, 2010). *Gdf10* localizes to the cerebellar VZ as early as E13.5 and by E15.5, *Gdf10*-positive cells actively migrate toward the pial surface, as part of a migration process that these cells undergo until postnatal day P7 (Yamada and Watanabe, 2002). From P7 onwards *Gdf10* expression in the PCL becomes progressively more restricted to fine band of BG cells located between the soma of Purkinje neurons (**Figures 2U**,**V**). Our knowledge of possible interaction partners of *Gdf10* is very limited and *Gdf10* null mutant mice develop normally (Zhao et al., 1999). However, in PC degeneration mice (pcd3J) *Gdf10* is reduced to 15% of the signal obtained from wild type littermates of the same age (4 postnatal months; Rong et al., 2004).

HUMAN CEREBELLAR DISORDERS RELATED TO DEFECTS AT THE ISTHMIC ORGANIZER

As we mentioned above, the cerebellum and its stereotyped circuitry, contributes not only to motor learning and correction of motor acts, but also to cognitive and emotional functions. Clumsiness and abnormal motor behavior have been well-documented in disorders such as autism and Asperger's syndrome (see Frith, 1991; Fatemi et al., 2012; Rogers et al., 2013), in dyslexia (Nicolson et al., 2001) and in schizophrenia (Owens et al., 1982). The cerebellum is ontogenetically and functionally heterogeneous, with cerebellar zones from different precursor domains (r0/r1 vermis or r1 cerebellar hemispheres) selectively interconnecting with different cerebral subsystems. In addition, the main cellular and molecular processes in cerebellar histogenesis are regulated by the same morphogenetic signals operating in other brain regions (Airey et al., 2001; Echevarría et al., 2003; Vieira et al., 2010; **Figures 3A**,**B**). Thus, it is not surprising to find developmental disorders that affect both different functional systems in the forebrain and the cerebellum. Advances in developmental genetics, neurobiology, molecular

biology, and neuroimaging have led to a better understanding of developmental disorders arose from the embryonic midbrain and hindbrain, (Barkovich et al., 2007, 2009). Although malformations of the hindbrain maybe the only recognized abnormality in individuals with mental retardation or autism (Soto-Ares et al., 2003; Courchesne et al., 2005), they are more commonly associated with malformations of the cerebrum. A contribution to our incomplete knowledge of the clinical consequences of hindbrain and cerebellar anomalies may be due to intrinsic difficulties in neuroimaging and anatomical complexity of the cerebral region.

The combination of a shortened midbrain and/or elongated pons is associated with an enlarged anterior vermis in humans presumably due to a rostral displacement of the IsO, with loss of mesencephalic tissue and gain of cerebellar tissue (**Figure 3C**). In fact, this malformation is presumed to result from *GBX2* predominance over *OTX2* and a consequent rostral shift of the IsO (Chizhikov and Millen, 2003; Barkovich et al., 2009). In the counterpart of this phenotype, Ballabio and co-workers (Quaderi et al., 1997) described opposite phenotype in the Opitz G/BBB syndrome (OS), a X-linked genetic anomaly that is caused by a loss of function of the *MID1* gene. Neuroimaging of human brain patients lacking this gene showed hypoplasia of the anterior cerebellar vermis (Pinson et al., 2004; Fontanella et al., 2008; **Figures 3C,F**). Concomitantly, *Mid1*-null mice show also vermis hypoplasia among other motor coordination defects (Lancioni et al., 2010). In these mice a rostralization of the mid-hindbrain constriction occurs together with a down-regulation of Fgf17, an important signal during the morphogenetic activity of the IsO (Xu et al., 2000; Zanni et al., 2011). Thus, the increasing of knowledge in basic embryology, genetics, and in cellular and molecular biology of the developing brain must be emphasized to prove the importance in recognizing, understanding, and classifying anomalies in human pathologies (Barkovich, 2012).

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Motor learning of mice lacking cerebellar Purkinje cells

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The cerebellum plays a key role in the acquisition and execution of motor tasks whose physiological foundations were postulated on Purkinje cells' long-term depression (LTD). Numerous research efforts have been focused on understanding the cerebellum as a site of learning and/or memory storage. However, the controversy on which part of the cerebellum participates in motor learning, and how the process takes place, remains unsolved. In fact, it has been suggested that cerebellar cortex, deep cerebellar nuclei, and/or their combination with some brain structures other than the cerebellum are responsible for motor learning. Different experimental approaches have been used to tackle this question (cerebellar lesions, pharmacological agonist and/or antagonist of cerebellar neurotransmitters, virus tract tracings, etc.). One of these approaches is the study of spontaneous mutations affecting the cerebellar cortex and depriving it of its main input–output organizer (i.e., the Purkinje cell). In this review, we discuss the results obtained in our laboratory in motor learning of both *Lurcher* (Lc/+) and *tambaleante* (*tbl/tbl*) mice as models of Purkinje-cell-devoid cerebellum.

Keywords: cerebellum, motor learning, *Lurcher, tambaleante*, Purkinje cells

INTRODUCTION

The cerebellum coordinates motor activities to be performed or already underway. In fact, cerebellar damage produces disturbance in movements and in body support. The relationship between cerebellum and motor learning was first suggested with the studies of Ramón y Cajal (1911), Dow and Moruzzi (1958), and Eccles et al. (1967). Dow and Moruzzi (1958) hypothesized that the cerebellum contributes to motor learning by determining how to perform accurate and correct movements. Thereafter, numerous studies have been devoted to analyzing the role of the cerebellum in perceptive and cognitive processes. Thus, the essential contribution of Marr, localizing the site of motor learning in the cerebellar cortex (Marr, 1969), and the later application of Marr's theory to the classical conditioning (Albus, 1971), whose physiological basis are directly related to *long-term depression* (LTD) mechanisms (Ito, 1989), defined the neuronal circuit involved in associative motor learning which remains accepted and discussed.

The anatomically highly refined organization of the cerebellum and its afferent/efferent pattern of projections from/to motor and premotor regions of cerebral cortex and spinal cord provides a paradigmatic substrate supporting its participation in motor behavior and learning (see Bernard et al., 2012). Furthermore, the analysis of the development of the cerebellar hemispheres and the expansion of the cerebral cortex in phylogeny also suggests the involvement of the cerebellum in cognitive functions (Leiner et al., 1986). The topography of the cerebellar cortex is closely related to deep cerebellar nuclei arrangement which also have different motor function according to the region of the cerebellum in which they are situated, and each nucleus controls a different aspect of the movement for the whole-body map (Thach et al., 1992; Thach, 1997).

Numerous studies have been performed in order to determine the role of the different cerebellar parts as a site of motor learning and/or memory storage. Among the authors who give a central role to the cerebellum, some point to the cerebellar cortex (Attwell et al., 2001; Chen et al., 1996), some to the deep cerebellar nuclei (Clark et al., 1992; Bracha et al., 2001), while others postulate the coordinated work of cerebellar and extra-cerebellar regions (Aou et al., 1992; Delgado-García and Gruart, 2002; Christian and Thompson, 2003; Koekkoek et al., 2003; Jiménez-Díaz et al., 2004; Porras-García et al., 2005, 2010; Sánchez-Campusano et al., 2007, 2009; Freeman and Steinmetz, 2011). Different experimental approaches, such as retrograde trace with virus (Morcuende et al., 2002), lesions, or pharmacological studies of cerebellar structures (Yeo et al., 1985a,b; Bracha et al., 1999; Christian and Thompson, 2003; Jiménez-Díaz et al., 2004), electrophysiological recordings from cerebellar cortex and nuclear neurons (Gruart et al., 2000; Porras-García et al., 2010), the study of cerebellar developmental disorders (Manto and Jissendi, 2012), and the use of mutant mice (Chen et al., 1996; Grüsser-Cornehls and Bäurle, 2001; Koekkoek et al., 2003, 2005; Porras-García et al., 2005, 2010), have been used to elucidate the function of the cerebellum. For this last approach, some mutations resulting in a total loss of Purkinje cells are very useful. Here, we summarize the results obtained in *tambaleante* and *Lurcher* mutant mice as models used for this purpose.

LURCHER MUTATION

The *Lurcher* mutation appeared spontaneously in 1954 in the mouse colony of the Medical Research Council Radiobiological

Research Unit at Harwell, England. In 1960, Phillips described motor-coordination problems associated to the *Lurcher* mutation. He also reported that this mutation was semi-dominant and the gene was localized on chromosome 6 (Phillips, 1960). The *Lurcher* mutation is caused by mutation in the δ2 glutamate receptor (GluRδ2; Caddy and Biscoe, 1979; Zuo et al., 1997). GluRδ2 is predominantly expressed in both Purkinje cells and several hindbrain cells (Araki et al., 1993; Lomeli et al., 1993; Mayat et al., 1995; Takayama et al., 1995, 1996; Landsend et al., 1997). Homozygous *Lurcher* mice (*Lc/Lc*) die after birth (P0) through a massive loss of mid- and hind-brain cells (Cheng and Heintz, 1997; Resibois et al., 1997). In contrast, the heterozygous *Lurcher* mouse (*Lc/+*) suffers cerebellar Purkinje cell death from the third and fourth day after birth (P3–P4) (Swisher and Wilson, 1977). From the 8th day after birth (P8), the Purkinje cell loss produces the degeneration of granule cells and olivary neurons (Caddy and Biscoe, 1979). Three months after birth, the *Lc/+* mouse has lost almost every Purkinje cell, some 90% of granule cells, and some 75% of the olivary neurons (Caddy and Biscoe, 1979; Wetts and Herrup, 1982; Heckroth and Eisenman, 1991; Norman et al., 1995; Wullner et al., 1995; Doughty et al., 2000).

Despite the motor problems that appeared in *Lc/+* mice in various motor tests performed (fall, rotarod, ladder, horizontal bar, eyeblink classical conditioning), they were able to learn new motor tasks, but the amplitude of the learned responses were significantly lower than in wild-type mice (Lalonde, 1994; Caston et al., 1995; Le Marec et al., 1997; Hilber and Caston, 2001; Porras-García et al., 2005) (**Figure 1**). However, the lesion of the interpositus nucleus prevented the generation of conditioned eyeblink responses in *Lc/+* and wild-type mice (Yeo et al., 1985a; Welsh and Harvey, 1989; Bracha et al., 1999; Jiménez-Díaz et al., 2004; Porras-García et al., 2010). Moreover, electrophysiological recordings of interpositus and red nuclei in *Lurcher* mice during the eyeblink classical conditioning suggest compensatory mechanisms in the absence of cerebellar cortex during performance of learned movements (Porras-García et al., 2010). These results suggest that deep cerebellar nuclear neurons (interpositus and dentate nuclei) may be involved more in the modulation and proper performance of ongoing conditioned responses than in their generation and/or initiation during learning processes (Gruart et al., 1997; Delgado-García and Gruart, 2002; Jiménez-Díaz et al., 2004).

TAMBALEANTE MUTATION

The *tambaleante* mutation (*tbl*) is a recessive mutation that appeared spontaneously in a DW/J-Pas background at the Pasteur Institute (Paris, France). This mutation affects cerebellar Purkinje cells, leading to their disappearance (Wassef et al., 1987). In the homozygous *tambaleante* (*tbl/tbl*) mutation, the degeneration of Purkinje cells begins from the second month of life—a date from which the Purkinje cell number decreases dramatically. When the *tbl/tbl* mouse is 1 year old, fewer than 1% of the cerebellar Purkinje cells survive (Dusart et al., 2006) (**Figure 2**). The gene related to the mutation is *Herc1*, which expresses a protein involved in the growth and maintenance of the cerebellar cytoarchitecture. Moreover, there seems to be a relationship between the increased levels of the mutated protein HERC1 and the autophagic death of the Purkinje cells in the *tambaleante* mouse (Mashimo et al., 2009).

The *tambaleante* (*tbl/tbl*) mutation develops an ataxic syndrome (Wassef et al., 1987; Rossi et al., 1995), with signs of tremor, unstable gait, and abnormal posture of hind limbs, similar to that in other mutated cerebella devoid of Purkinje cells (**Table 1**). However, the phenotype of the *tbl/tbl* mouse and the regressive phenomena that will take place "en cascade" as in other Purkinje-cell-mutated cerebella (Sotelo and Changeux, 1974a; Caddy and Biscoe, 1979) are not completely understood. Thus, data are still not available as to whether the *tbl/tbl* mutation could affect only cerebellar Purkinje cells or whether other cerebellar and extracerebellar neuronal cell populations would also be affected directly or indirectly by the mutation. Despite the lack of concrete data on the extent of the *tbl/tbl* mutation, the late onset of complete Purkinje-cell degeneration with respect to other mutations (**Table 1**), once all cerebellar circuits have developed normally, makes it a perfect model for studying the cerebellar involvement in various motor learning tasks.

Recent studies carried out in our lab show that the *tbl/tbl* mouse seems not to have its motor learning capabilities completely affected (**Figure 1**). Thus, although slower than wild-type mice, *tbl/tbl* mice perform both fall and horizontal bar test successfully (**Figures 1A,B**), learning consistently from the first to last session. However, *tbl/tbl* mice were unable to adapt their motor responses in the vertical pole test, in which they systematically failed to learn through the four sessions (**Figure 1C**).

Purkinje cell loss elicits a series of compensatory structural changes in the main cerebellar output (i.e., from deep cerebellar and vestibular nuclei). The diminution on Purkinje cell inhibitory input leads to changes in these neuronal populations that have been closely related to mutant behavioral phenotypes. Among these neural responses, an increase in the parvalbumin (Parv) was consistently found in cerebellar and vestibular nuclei after spontaneous or surgical Purkinje cell deprivation (Grüsser-Cornehls and Bäurle, 2001). In DW/J-Pas wild-type mice, deep cerebellar and vestibular nuclei neuronal somata are Parv- (**Figure 2D**), while small Parv+ terminal endings are present (**Figure 2E**). As in the *Lc/+* mutation (Grüsser-Cornehls and Bäurle, 2001), Parv+ somata are found through all deep cerebellar nuclei in *tbl/tbl* mice (**Figures 2F,G**). Parv immunoreactivity of presynaptic boutons is also different in *tbl/tbl* deep cerebellar nuclei, and—together with small boutons (**Figure 2I**)—there is an increase in the presence of larger Parv+ endings (**Figure 2H**). Therefore, Parv immunoreactivity of *tbl/tbl* mice is similar to that observed in other Purkinje-cell-deprived mutations, reinforcing the relationships between motor behavior and Parv expression in deep cerebellar and vestibular nuclei (Grüsser-Cornehls and Bäurle, 2001).

Despite the similarities in the structural changes suffered by the different Purkinje-cell-devoid mutant mice sharing a similar motor behavior (**Table 1**), there are subtle differences in the motor response of the two strains analyzed here. Thus, while no differences were found in the fall test (**Figure 1A**), *tbl/tbl* mice seemed to perform the horizontal bar test more easily than did *Lc/+* ones (**Figure 1B**). In contrast, *tbl/tbl* mice were consistently unable to successfully perform the vertical

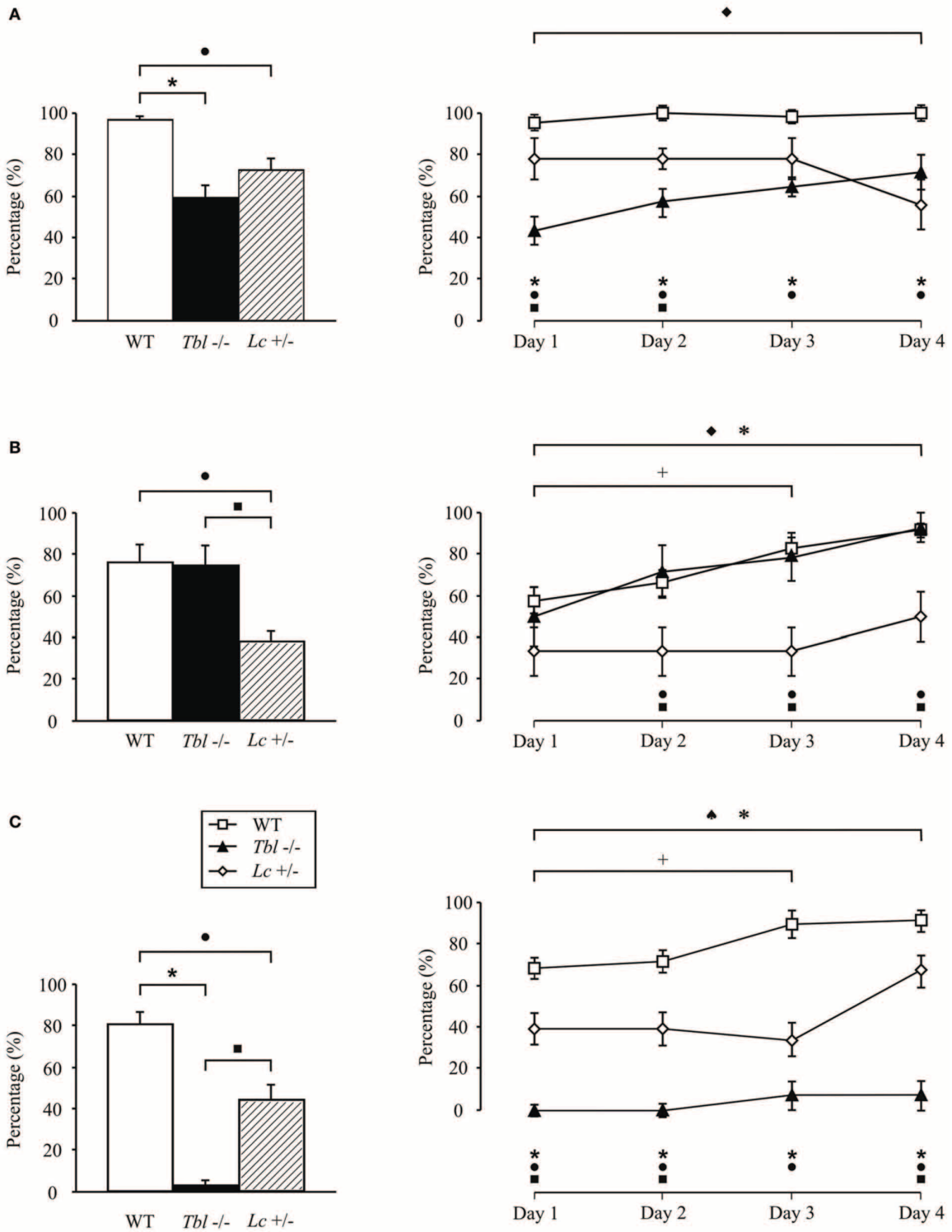

FIGURE 1 | Comparison of motor activity and motor learning in *tambaleante* (*tbl*$^{-/-}$), Lurcher (*Lc*/+), and wild-type (WT) mice evaluated in various motor tasks: fall (A), horizontal bar (B), and vertical pole (C) performed twice a day during 4 days. Significant differences were found between groups in the percentage (%) mean values (left) [One-Way ANOVA *F*-test, $F_{(6,\ 1158)} = 17.50$ (fall); $F_{(6,\ 1158)} = 8.45$ (horizontal bar); $F_{(6,\ 1158)} = 45.28$ (vertical pole), $P < 0.05$] as well as in the temporal evolution for each of the tests (right). [Two-Way ANOVA *F*-test, $F_{(4,\ 360)} = 2.41$ (fall); $F_{(4,\ 360)} = 0.56$ (horizontal bar); $F_{(4,\ 360)} = 1.62$ (vertical pole), $P < 0.05$]. ♦, significant differences between *tambaleante* mice; +, between different sessions of wild-type animals; ♠, between *Lurcher* mice; ∗, between *tambaleante* and wild-type mice; ■, between *tambaleante* and *Lurcher* animals; and •, between *Lurcher* and wild-type mice. *Lurcher* mice data collected from Porras-García et al. (2005).

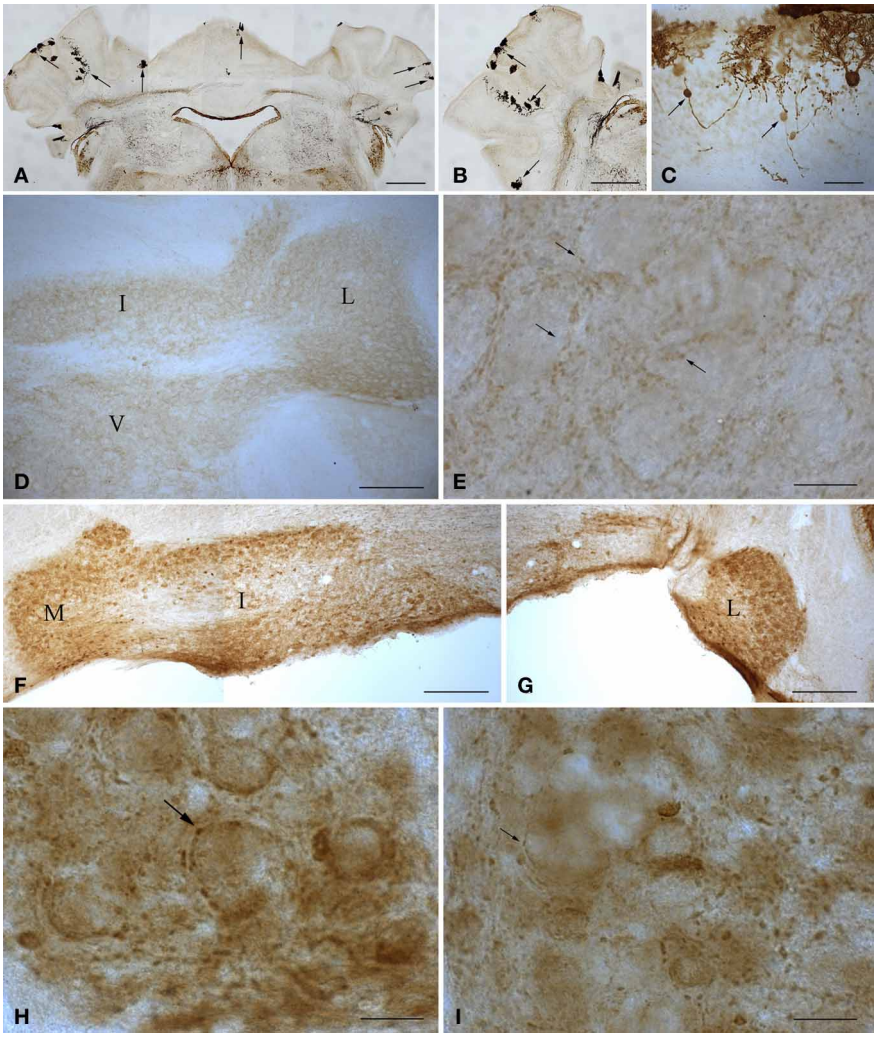

FIGURE 2 | Microphotographs of coronal (A, D–I) and sagittal (B,C) sections immunostained with anti-calbindin (A–C) and anti-parvalbumin (D–I) antibodies illustrate the main features of the cerebellum of wild type and 1-year-old *tambaleante* mice. Scarce Purkinje cells remain throughout the cortex (**A–B**, arrows), preserving a mirror location on both sides of the cerebellar cortex (**A**, arrows). Axons of degenerating Purkinje cells show typical axonal torpedoes (**C**, arrows). Parvalbumin immunoreactivity is restricted to small endings (**E**, arrows), and is absent in both deep cerebellar and vestibular nuclei neuronal somata **(D)**, in wild-type cerebellum. In *tambaleante* cerebellum, parvalbumin immunoreactivity is present in neuronal somata of deep cerebellar nuclei **(F,G)**, and in large (**H**, arrow) and small (**I**, arrow) terminal endings. I, L, and M, interposed, lateral, and medial cerebellar nuclei. V, vestibular nuclei. Bar = 500 μm **(A,B)**, 200 μm **(D,F,G)**, 30 μm **(C)**, and 20 μm **(E,H,I)**.

pole test, while *Lc*/+ mice did (**Figure 1C**). These differences in motor learning could be due to dissimilarities between the two mutant mice in the structural changes in cerebellar connectivity as the result of Purkinje cell loss. A possible explanation of these differences could reside in the different temporal onset of Purkinje-cell degeneration, as *tbl/tbl* is the only mutation that losses all Purkinje cells once the cerebellar circuits have developed normally (Wassef et al., 1987; Rossi et al., 1995; Dusart et al., 2006) (**Table 1**). A detailed analysis of the *tbl/tbl* cerebellum regarding the total amount of granule cells after Purkinje cell loss, and the cerebellar cortico-nuclear relationships at the beginning of the mutation effect, would explain these motor learning differences. Accordingly, the comparison of amounts of GABAergic input to cerebellar deep nuclei between *Lc*/+ and *tbl/tbl* mice could help to solve the question (see Grüsser-Cornehls and Bäurle, 2001). However, it is noteworthy that genes leading to Purkinje cell degeneration also affect other brain regions, and that ataxic symptoms and motor behavior are most severe in mutant mice whose brain is widely affected (**Table 1**). Hence, the analysis of *tbl/tbl* brain areas involved in motor behavior and the possible compensatory processes taking place after the loss of Purkinje cells when all motor circuitry is fully developed would explain the differences in *tbl/tbl* motor behavior.

CONCLUSION

Various studies give the cerebellar cortex an important role in motor learning. However, the results obtained in our laboratory,

Table 1 | Main mutations affecting cerebellar Purkinje cells (Pc).

Mutation	Onset of Pc degeneration	End of Pc degeneration	Size of Pc degeneration	Cerebellar and pre-cerebellar associated cell degenerations	Associate neural degenerative changes	Motor impairment	References
Leaner (*tg*^{1a}/tg^{1a})[1]	P15–40	5, 6 month	Alternate Pc bands with bands devoid of Pc[2]	Golgi neurons[3], granule cells[3], inferior olivary neurons[4]	Hippocampus[5, 6]	Most severe that in other mutations[6]	[1]Dickie (1962) [2]Heckroth and Abbott (1994) [3]Herrup and Wilczynski (1982) [4]Zanjani et al. (2004) [5]Alonso et al. (2008) [6]Grüsser-Cornehls and Bäurle (2001)
Lurcher (*Lc*/+)[1]	P3–4	4 month	Complete	Inferior olivary neurons[2], granule cells[2]	No data available	Mild[3]	[1]Phillips (1960) [2]Caddy and Biscoe (1979) [3]Grüsser-Cornehls and Bäurle (2001)
Nervous (*nr/nr*)[1]	P23	P50	Alternate Pc bands with bands devoid of Pc[2]	Inferior olivary neurons[3]	Retinal photoreceptors[4]	Mild[5]	[1]Sidman and Green (1970) [2]Wassef et al. (1987) [3]Zanjani et al. (2004) [4]Mullen and LaVail (1975) [5]Grüsser-Cornehls and Bäurle (2001)
Pogo (*pogo/pogo*)[1]	P120	–	Vermal Pc[2]	No data available	No data available	Mild[1, 2]	[1]Lee and Jeong (2009) [2]Jeong et al. (2000)
Purkinje cell degeneration (*pcd/pcd*)[1]	P15	P45	Complete	Inferior olivary neurons[2], granule cells[3], cerebellar nuclei neurons[4]	Retinal photoreceptors[1], olfactory bulb mitral cells[1], thalamic neurons[5]	Mild[6]	[1]Mullen et al. (1976) [2]Ghetti et al. (1987) [3, 4]Triarhou et al. (1985); Triarhou et al. (1987) [5]O'Gorman (1985) [6]Grüsser-Cornehls and Bäurle (2001)
Reeler (*rl/rl*)[1]	P0	P15	≤50% with ectopic remaining Pc[2]	GABAergic interneurons[3], granule cells[4], unipolar brush cells[4]	Hippocampus[5], neocortex[5]	Mild	[1]Falconer (1951) [2]Heckroth et al. (1998) [3]Takayama (1994) [4]Ilijic et al. (2005) [5]Park and Curran (2008)
Staggerer (*sg/sg*)[1]	P0	P24	75% with ectopic remaining Pc[2]	All granule cells[2], inferior olivary neurons[3]	Hippocampus[4], olfactory bulb[5]	Most severe than in *Lc*, *nr*, or *pcd*[6]	[1]Sidman et al. (1962) [2]Sotelo and Changeux (1974a) [3]Zanjani et al. (2007) [4]Yi et al. (2010) [5]Deiss et al. (2001) [6]Grüsser-Cornehls and Bäurle (2001)
Tambaleante (*tbl/tbl*)[1]	P60	4–6 month	Complete[1]	No data available	No data available	Mild, like *Lc* or *pcd*[2, 3]	[1]Wassef et al. (1987) [2]Rossi et al. (1995) [3]present observations
Weaver (*wv/wv*)[1]	P0	P14	25% with great alteration of the dendritic trees of remaining Pc[2, 3]	Granule cells[2, 3]	Hippocampus[4], substantia nigra pars compacta[5]	Most severe than in *Lc*, *nr*, *pcd, or sg*[6]	[1]Lane (1965) [2]Sotelo and Changeux (1974b) [3]Sotelo (1980) [4]Sekiguchi et al. (1995) [5]Schmidt et al. (1982) [6]Grüsser-Cornehls and Bäurle (2001)

using two Purkinje-cell-deprived mutant mice, show that while this structure is not essential in learning, its absence disturbs the performance and the magnitude of the learned response (Porras-García et al., 2005, 2010). This is not so in the case of the interpositus nucleus of the cerebellum, in which any injury causes a total lack of learning (Porras-García et al., 2010). In accord with our results and those of some other authors, it seems motor learning must be due to coordinated work between several cerebellar and extra-cerebellar structures.

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Profound morphological and functional changes of rodent Purkinje cells between the first and the second postnatal weeks: a metamorphosis?

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Between the first and the second postnatal week, the development of rodent Purkinje cells is characterized by several profound transitions. Purkinje cells acquire their typical dendritic "espalier" tree morphology and form distal spines. During the first postnatal week, they are multi-innervated by climbing fibers and numerous collateral branches sprout from their axons, whereas from the second postnatal week, the regression of climbing fiber multi-innervation begins, and Purkinje cells become innervated by parallel fibers and inhibitory molecular layer interneurons. Furthermore, their periods of developmental cell death and ability to regenerate their axon stop and their axons become myelinated. Thus a Purkinje cell during the first postnatal week looks and functions differently from a Purkinje cell during the second postnatal week. These fundamental changes occur in parallel with a peak of circulating thyroid hormone in the mouse. All these features suggest to some extent an interesting analogy with amphibian metamorphosis.

Keywords: Purkinje cell, development

INTRODUCTION

Understanding how brain complexity develops is one of the challenges of neurobiology. Neurons and glia mature in a succession of transitions between cell states. The orchestration of these processes for different cell types requires the tight regulation of both intrinsic and extrinsic factors. Although each cell type undergoes its own program of differentiation, the timing of differentiation must be coordinated between different cell types. Thus, during brain formation, the regulation of cell number and the precise timing of differentiation require the interplay between intrinsic programs of development and extrinsic factors.

The rodent cerebellum is an attractive model system for the study of cell differentiation, as it consists of a small number of neuronal types that have been morphologically and molecularly well characterized (for review see Armengol and Sotelo, 1991; Sotelo, 2004). Three major types of neurons with very different properties are found in the cerebellar cortex: Purkinje cells, granule cells, and GABAergic interneurons (basket, stellate, and Golgi neurons). They differ in their function, morphology, origin, migration routes, and differentiation timing. Neurogenesis in the cerebellum extends over a protracted period of time, between embryonic day 10.5 and postnatal day 15 in mice, and parallels glial cell proliferation and differentiation. As a consequence, successive steps in the differentiation of several cerebellar cell types are spread over a long time period.

Purkinje cells, some of the largest neurons in the central nervous system, have a central place among the different cell populations of the cerebellar cortex. Purkinje cells are the only efferents of the cerebellar cortex, mainly sending inhibitory projections to the deep cerebellar nuclei, and thus constitute the sole output for all motor coordination and learning from the cerebellar cortex. Many fundamental concepts of modern neuroscience have been established by a focus on this spectacular cell type (see review Sotelo, 2004). They are the targets of numerous naturally occurring neurological mutations (Dusart et al., 2006; Sajan et al., 2010). 5-hydroxy-methyl-cytosine, a modified nucleotide which function remains enigmatic, was first discovered in Purkinje cell DNA (Kriaucionis and Heintz, 2009). Furthermore, Purkinje cells, being the only output of the cerebellar cortex, control the function of the cerebellum. Thus, when Purkinje cells are affected, it is very easy to detect through behavioral phenotypes. Furthermore, their typical location and morphology make them very easy to study. For these reasons, they have been more frequently studied than other neurons and consequently they have been more often detected as implicated in pathologies. The Purkinje cell constitutes thus a classical model to study a number of aspects of neuronal differentiation.

Purkinje cells are the first neuron of the cerebellar cortex to be generated; they are born during the early fetal development (embryonic days 11–13 in mice, Miale and Sidman, 1961; Carletti and Rossi, 2008); they end their phase of migration 2 or 3 days before birth and their axons reach their targets in the deep cerebellar nuclei by the end of the fetal period (embryonic day 17, Eisenman et al., 1991). Between embryonic day 19 and the day of birth, Purkinje cells receive their first climbing fiber synapses (Mason et al., 1990; Chedotal and Sotelo, 1992). However, the development of mouse Purkinje cells occurs largely during the first three postnatal weeks of life, during which Purkinje cells

develop dendrites and establish synaptic connections (Sotelo and Dusart, 2009). Although it largely coincides with granule cell proliferation and maturation of GABAergic interneuron precursors in neighboring cerebellar layers, the postnatal Purkinje cell maturation process is probably also governed by an intrinsic genetic program.

The aim of this review is to focus on the events that occur during the transition between the first and the second postnatal week and to investigate to which extent these events can be driven by the peak of circulating thyroid hormone that occurs at the end of the first postnatal week in the mouse.

THE END OF THE FIRST POSTNATAL WEEK: A PERIOD OF TRANSITION IN PURKINJE CELL DIFFERENTIATION

DENDRITIC DIFFERENTIATION

Morphological transition at the end of the first postnatal week

Shortly after birth, cerebellar Purkinje cells have a bipolar shape reminiscent of their migratory morphology, with a primary dendrite at their apical pole and an axon at the basal pole. Ramon y Cajal was the first to describe this stage, which he called "phase of fusiform corpuscle" (Cajal, 1926). In 1991, Armengol and Sotelo described two types of fusiform stages in the rat: a simple fusiform corresponding to Cajal's description, and a complex fusiform, presenting a more elaborated dendritic tree in which some collateral branches develop from the primary dendrites (Armengol and Sotelo, 1991). Around postnatal day 3 in rats, the primary dendrite has regressed, in parallel with the emergence of numerous perisomatic dendritic processes. At this stage, the Purkinje cells are not polarized, and Cajal called this stage "stellate." Several different stellate stages have since been more precisely described (Armengol and Sotelo, 1991). At least two different morphological types can be distinguished: a true stellate form, in which the processes are thin, long, and without spines, and an atrophic stage, in which the processes are short. A third intermediate type has also been described in which few (two or three) dendrites emerge from the soma at the apical pole. Interestingly, the small dendrites of this later stage have spines. All these stages can be visualized in parallel during the first postnatal week, as the development of the cerebellar cortex is not synchronous: different developmental stages can coexist within a same lobule and among neighboring Purkinje cells (Armengol and Sotelo, 1991). Only video-microscopy would show whether all the Purkinje cells go through these different stages and in which order. However, the study of the proportions of cells in the different stages over time either *in vivo* in rats (Armengol and Sotelo, 1991) or *ex vivo* in organotypic cultures (Boukhtouche et al., 2006b; Poulain et al., 2008) suggests that the Purkinje cells pass through these different stages in the described order (Sotelo and Dusart, 2009).

At the beginning of the second postnatal week, the Purkinje cells have a single stem segment at their apical pole. From this time, a "cerebellist" can easily recognize the early form of the future mature Purkinje cell dendritic tree. One particularity of this dendritic tree is that the growth and the ramification occur in the sagittal plane (Kaneko et al., 2011). Thus, as described by Cajal, mature Purkinje cell dendritic tree resembles an "espaliered" fruit tree (Cajal, 1911). Larramendi proposed that this transition from multiple dendritic trees to a single one could be the result of the sudden drop of the Purkinje cell nucleus toward the basal pole (Larramendi, 1969). In parallel with this transition, Purkinje cell somata merge from multiple irregular rows into a single layer. During the second postnatal week, and up to the end of the third postnatal week, the dendritic tree grows first wider and then taller (Berry and Bradley, 1976).

Morphological changes: cell-autonomous versus non-cell-autonomous processes

The first dendritic differentiation phases are likely to be driven by intrinsic Purkinje cell developmental programs. The very few purified newborn mouse Purkinje cells (0.2%) that survive in dissociated culture have smaller dendrites after 21 days *in vitro* than after 4 days *in vitro*, suggesting that the regressive events occur *in vitro* (Baptista et al., 1994). In these culture conditions, Purkinje cells never acquire their typical dendritic form. In organotypic culture, Purkinje cells grown in the absence of climbing fiber present similar dendritic developmental phases as those described *in vivo* (Boukhtouche et al., 2006b; Poulain et al., 2008), suggesting that climbing fibers are not necessary for the general sculpting of the dendritic trees. However, in the absence of climbing fibers, the size of the dendritic tree was reduced, due to a decrease in the total number of dendritic segments whereas individual segment lengths were largely unaltered (Bradley and Berry, 1976). In contrast, the study of experimental models or mutant mice in which the development of parallel fibers is impeded has revealed that parallel fibers are very important for the growth and planar arrangement of the mature dendritic tree (for review see Sotelo and Dusart, 2009). Thus, although the first postnatal phases of Purkinje cell dendritic differentiation are likely to be intrinsic, the later phases occurring from the second postnatal week dependent on the environment.

An interesting example of the importance of intrinsic factors has been described for the nuclear receptor RORα, which is deleted in *staggerer* mouse (Hamilton et al., 1996). The effect of this mutation on Purkinje cells has been long known (Sidman et al., 1962; Boukhtouche et al., 2006a; Gold et al., 2007). More recently, the role of RORα in the first stages of Purkinje cell dendritic development has been studied using lentiviral RORα overexpression in organotypic culture of newborn cerebellar slices (Boukhtouche et al., 2006b). In this model, 58% of RORα transduced Purkinje cells are already in an atrophic stage after 3 days of culture, while 94% of control Purkinje cells are still in the fusiform stages. After 5 days in culture, 57% of the transduced Purkinje cells are already in a mature stage and present numerous spines. These results indicate that the overexpression of RORα first promotes the regression of the primary dendritic tree and then accelerates dendritic development (Boukhtouche et al., 2006b). Later over-expression does not alter Purkinje cell morphology, suggesting a restriction of the developmental function of RORα to early stages (Boukhtouche et al., 2006b). Although the growth of the characteristic form of Purkinje cells is dependent of the environment (for review see Sotelo and Dusart, 2009), the factors that drive its specific form are still unknown.

TRANSITION OF SYNAPTIC COMPONENTS AT THE END OF THE FIRST POSTNATAL WEEK

General description of the development of synaptic connections on Purkinje cells

Purkinje cells are at the center of the cerebellar neuronal circuit. Each Purkinje cell receives up to 200 000 synapses and transmits the integrated signal to the deep nuclei. The innervation of Purkinje cells undergoes profound modifications during the first two postnatal weeks. As it has been recently reviewed, cerebellar developing circuits typically differ substantially from their mature counterparts, which suggests that development may not simply involve synaptic refinement, but rather involves restructuring of key synaptic components and network connections, in a manner reminiscent of metamorphosis (van Welie et al., 2011).

Purkinje cells establish functional synapses with deep nuclear neurons between postnatal days 2 and 6 (Gardette et al., 1985) and at this time Purkinje cell axons grow many collaterals (Gianola et al., 2003). These recurrent axon collaterals underlie facilitating synapses between cerebellar Purkinje cells (Orduz and Llano, 2007). Interestingly, the Purkinje–Purkinje connection is asymmetric and provides a robust substrate for propagating waves of activity in the developing, but not adult, cerebellum (Watt et al., 2009).

During the first postnatal week, Purkinje cells are contacted by the presynaptic inputs of glutamatergic climbing fibers, the olivocerebellar afferents (Mason et al., 1990; Chedotal and Sotelo, 1992, 1993; Morara et al., 2001). Interestingly during this period, Purkinje cells pass through a phase of climbing fibers multi-innervation (Crepel et al., 1976; Mariani and Changeux, 1981; Kano and Hashimoto, 2009 for review). Some mossy fibers that in adult innervate granule cells can also transiently innervate Purkinje cells (Mason and Gregory, 1984; Takeda and Maekawa, 1989; Kalinovsky et al., 2011). In parallel, some GABAergic axon terminals abut on Purkinje cell somata (Sotelo, 2008; Ichikawa et al., 2011).

At the end of the first postnatal week, the supernumerary climbing fibers begin to be eliminated and the remaining one translocates and synapses onto the proximal dendritic compartment of PCs. The parallel fibers (the axons of granule cells) make synapses at the more distal part of Purkinje cell dendritic tree. In parallel, the different GABAergic interneurons start to innervate specific parts of the Purkinje cell (soma or dendritic tree) (Sotelo, 2008; Ichikawa et al., 2011). Interestingly, during the second postnatal week, considerable fraction of Purkinje somatic spines is succeeded from glutamatergic climbing fibers to GABAergic Basket fibers, in parallel with the switching of postsynaptic receptor phenotypes (Ichikawa et al., 2011).

Thus, at the end of the first postnatal week, the development of Purkinje cells is marked by increase of spinogenesis, synaptogenesis with parallel fibers and GABAergic molecular interneurons (Sotelo and Dusart, 2009; van Welie et al., 2011). In parallel with these events that will continue up to the third postnatal week, there is a transition between depolarizing and hyperpolarizing GABA, and the regression of climbing fiber multi-innervation occurs.

Transition between depolarizing and hyperpolarizing GABA

Based on two independent sets of experiments (calcium imaging using fura-2 loaded Purkinje cells and perforated-patch recordings) Eilers et al. demonstrated a depolarizing action of GABA on immature Purkinje cells (Eilers et al., 2001). They showed that the transition from depolarization to hyperpolarization occurs around postnatal day 6 (P6) in rats. Interestingly, GABA-mediated Ca^{2+} signaling was never detected in Purkinje cells with more elaborate dendritic trees (aged P8/9). The depolarizing action of GABA has also been observed in Purkinje cells from 3 day-old mice (Rakotomamonjy et al., 2011). Thus Purkinje cells, like some other neuronal populations, exhibit GABA-mediated depolarization during early postnatal stages of life (for review see Ben-Ari et al., 2007). It is thought that GABA depolarizes immature neurons because of a "reversed" chloride gradient in a wide range of neuronal types and animal species (Ben-Ari et al., 2007). The chloride accumulation in immature neurons can be due either to the early expression of transporters such as the Na-K-Cl co-transporter (NKCC) which accumulates chloride within the cell and/or the lack of expression of co-transporters such as K-Cl transporter (KCC) that export the chloride out of the cell (Delpire, 2000). For the majority of the neurons, the expression of KCC2 increases indeed at the end of the first postnatal week in rodents (for review see Ben-Ari et al., 2007). Surprisingly, Purkinje cells express KCC2 very early during development (Mikawa et al., 2002; Takayama and Inoue, 2007), but the intracellular chloride concentration can be regulated by other factors, such as the expression of WNK family kinases (Rinehart et al., 2011). How the intracellular chloride concentration is regulated within immature Purkinje cells is still an open question, and it is, therefore, not understood how the transition between GABA depolarization to hyperpolarization would be triggered in this neuron.

Multi-innervation of Purkinje cells by climbing fibers during the first postnatal week

In the adult, each Purkinje cell receives synapses from only one climbing fiber (mono-innervation). However, just after birth, around P3 in the rat, several climbing fibers converge and synapse onto the same Purkinje cell body, so that most Purkinje cells are shown to be initially innervated by multiple climbing fibers (Crepel et al., 1976; Mariani and Changeux, 1981). The peak of multi-innervation is around P5, and the regression of the multi-innervation starts at the end of the first postnatal week. In parallel, there is a translocation of the climbing fibers from the soma to the emergent dendritic tree (Cajal, 1911) although these two events can be dissociated. From this time until the end of the third week, one climbing fiber input is strengthened while supernumerary climbing fibers are weakened and finally eliminated, resulting in mono-innervation of Purkinje cells in the mature system (Hashimoto and Kano, 2003; Hashimoto et al., 2009). As reviewed recently, the phase of synaptic elimination can be divided in two phases: the first is between P7 and P12, and is independent of the parallel fibers; the second phase depends on parallel fibers [for review see (Kano and Hashimoto, 2009)]. Interestingly, it has been shown using co-culture and grafting experiments that climbing fiber synapse elimination occurs

only during a Purkinje-cell-dependent critical period (Gardette et al., 1990) and triggers indelible processes that prevent synapse competition in the mature system (Letellier et al., 2007, 2009). Whereas numerous actors (such as mGluR1, IGF, BDNF etc.) have been shown to be involved in the second phase of climbing fiber elimination [for review see (Kano and Hashimoto, 2009)], the mechanisms of the first phase at the transition between multi-innervation and regression of this multi-innervation are less understood. The progressive replacement of full-length TrkB by its truncated form on terminal climbing fibers at the end of the first postnatal week is likely to be involved in this process (Sherrard et al., 2009).

DEVELOPMENTAL PURKINJE CELL DEATH ENDS AT THE END OF THE FIRST POSTNATAL WEEK

Evidence for developmental Purkinje cell death

It took a long time for the importance of developmental cell death to be recognized (Ameisen, 2002), and even longer in the case of the Purkinje cell. During the intense phases of cell proliferation, cell death is a counterintuitive notion, and cell death by apoptosis is rapid (about 20-fold faster than proliferation) and difficult to observe directly. Thus, the best way to reveal neuronal cell death during development is to count a population of neurons at different time points. This implies the identification of a neuronal population with specific markers, which were lacking for early Purkinje cells (Madalosso et al., 2005; Dusart et al., 2006). However, numerous early indirect measures suggested that Purkinje cells pass through a phase of programmed cell death during their early development. Some Purkinje cells present pycnotic characteristics in the cerebellar primordium from embryonic day 15–16 (in mouse or chicken; Bertossi et al., 1986). A small number of TUNEL-positive or activated-Caspase3-positive Purkinje cells were observed in the P3–4 mouse cerebellum (Kitao et al., 2004; Marin-Teva et al., 2004). Jankowski et al. (2009) more precisely described the temporal and spatial distribution of pyknotic Purkinje cells during postnatal mouse development, observing dying Purkinje cells during the first postnatal week with a peak at P3, and very few if any pyknotic Purkinje cells after P9.

Furthermore, overexpression of the anti-apoptotic *bcl-2* gene at various stages of mouse development, and knock-out of the pro-apoptotic *bax* gene provided indirect indications of the existence and periods of programed developmental Purkinje cell death. Counting numbers of adult Purkinje cells shows a 40% increase in the transgenic overexpressing bcl-2 either from embryonic day 13 (E13) and a 27% increase if bcl-2 is overexpressed from P0, as well as a 30% increase for the bax deficient mice compared to wild types (Zanjani et al., 1996; Fan et al., 2001). In contrast, the expression of the human *Bcl-2* gene after P7 (using a *L7-HuBcl2* transgene, selectively expressed in Purkinje cells) did not change the total number of Purkinje cells, suggesting that the period of Purkinje developmental death ends before P7 (Goswami et al., 2005).

In organotypic culture, good survival is obtained when the cerebellum is explanted between E19 and P0, or after P10. By contrast, the great majority of the Purkinje cells die by apoptosis when the cultures are prepared from cerebellum between P1 and P8, with a maximum of death observed between P3 and P5 (Dusart et al., 1997; Ghoumari et al., 2000, 2002). Thus Purkinje cells are more vulnerable to the culture conditions between P1 and P8. Interestingly during this period, they are also more vulnerable to the noxious effects of alcohol (Pierce et al., 1999). This phase of high vulnerability has been proposed to reflect a period of programmed cell death (Dusart et al., 2005).

All these results suggest the existence of two periods of programmed Purkinje cell death, a first period during the embryonic life between E13–15, and a second period between P3 and P5 (Zanjani et al., 1996). Due to the identification of early Purkinje cell specific markers, it is likely that developmental Purkinje cell death will soon be re-evaluated. It is remarkable that the period of developmental cell death ends for mouse Purkinje cells at the end of the first postnatal week.

Purkinje cell survival factors

The program of cellular death is generally engaged by default when a mammalian cell is deprived of survival signals released by other cells (Raff et al., 1994). In contrast to other cell types, the vast majority of neurons are not renewed throughout the life of individuals. The neuronal periods of programmed cell death must be, therefore, tightly regulated. During development, neurons depend on trophic factors released by either their targets or their afferents for their survival (Oppenheim, 1991). According to the neurotrophic theory, this dependence allows the adjustment of numbers of neurons with their targets or afferent fibers.

During development, Purkinje cells, like other neurons, are dependent for their survival on signals produced by themselves and by other cellular types. The survival of dissociated and purified Purkinje cells *in vitro* increases by 14-fold in the presence of astrocytes, and by 32-fold in the presence of granule cells (Baptista et al., 1994). During their maturation, Purkinje cells express the neurotrophin receptors: LNGFR, TrkC, and TrkB (Yan and Johnson, 1988; Cohen-Cory et al., 1989; Lindholm et al., 1993; Minichiello and Klein, 1996; Velier et al., 1997). In parallel, the mRNA encoding neurotrophin-3 (NT-3) is abundant in rat granule cells between P5 and P20 and then replaced by brain-derived neurotrophic factor (BDNF) mRNA (Lindholm et al., 1993; Rocamora et al., 1993; Gao et al., 1995). In addition, Purkinje cells produce both insulin-like growth factor (IGF-1) and its receptor during their postnatal development (Bartlett et al., 1991; Bondy et al., 1992; Garcia-Segura et al., 1997). Interestingly, some of the IGF-1 present in the cerebellar cortex is transported by the climbing fibers from inferior olivary neurons to the Purkinje cells (Nieto-Bona et al., 1995). Furthermore, the deep nuclear neurons, the target of Purkinje cells, also express IGF-1 (Bondy et al., 1992). The mRNA and protein of Glial cell line-derived neurotrophic factor (GDNF) receptor are present in Purkinje cells during development (Burazin and Gundlach, 1999).

After the period of programmed cell death, neurons can survive in the absence of trophic factors or their targets: postnatal sympathetic neurons and septo-hippocampal cholinergic neurons gradually lose their dependency on Nerve Growth Factor or on their targets for survival (Lazarus et al., 1976; Sofroniew et al., 1990, 1993; Svendsen et al., 1994; Orike et al., 2001). Similarly, adult Purkinje cells survive for very long periods in the absence

of connections with their main target (deep nuclear neurons) and afferents (climbing fibers) (Dusart and Sotelo, 1994; Morel et al., 2002). From the pattern of expression of trophic factors in the developing cerebellum, it is difficult to propose a model to explain why Purkinje cells become independent from the presence of their targets at the end of the first postnatal week. It is likely that this target independence is the consequence of other processes than trophic factor availability.

Organotypic culture has been successfully used to unravel the role of different molecules (Ghoumari et al., 2000, 2002, 2003, 2006; Rakotomamonjy et al., 2011; Repici et al., 2011) and of microglial cells (Marin-Teva et al., 2004) in developmental Purkinje cell death. During the first week of postnatal life, mouse Purkinje cells show high expression of Caspase-3 mRNA (de Bilbao et al., 1999), suggesting that they are competent to die. The role of Lifeguard in Purkinje cell survival has been recently underlined (Hurtado de Mendoza et al., 2011). The mechanisms responsible for closing the period of neuronal target dependence have been studied in depth at the level of the apoptotic pathway (Putcha et al., 2000; Orike et al., 2001; Wright and Deshmukh, 2006; Wright et al., 2007; Vaughn and Deshmukh, 2008; Kole et al., 2011), but it is likely that for the moment, we have only seen the tip of the iceberg.

OTHER PROCESSES WITH A "TRANSITION PHASE" AT THE END OF THE FIRST POSTNATAL WEEK

Glial cell differentiation

The form and cytoskeletal content of Bergmann glia, a cerebellum-specific type of radial glia whose nuclei are in the Purkinje cell layer, change considerably during embryonic and postnatal development. Their content in glial fibrillary acidic protein increases at the end of the first postnatal week (Bovolenta et al., 1984).

Oligodendrocyte precursor cells (OPCs) are already present in the cerebellum at embryonic stages (Levine et al., 1993), but myelination in the mouse cerebellum begins only at the end of the first postnatal week (Foran and Peterson, 1992). Thus, for at least one week, the OPCs are in the presence of Purkinje cell axons but their differentiation is somehow inhibited. The synchronization of cell development is particularly important for cell types that have strong interactions, the case for oligodendrocytes and neurons. OPCs start to differentiate if a mitogenic stimulus is removed or a differentiation stimulus is added, and conversely this cell differentiation is inhibited in the presence of a mitogenic stimulus and the absence of a differentiation stimulus (Durand and Raff, 2000). This delayed differentiation period can be reproduced *in vitro* using organotypic culture (Bouslama-Oueghlani et al., 2003). However, how OPC and Purkinje cell differentiation are synchronized remains an open question.

Axon regeneration

Axonal regeneration in the mammalian CNS is a characteristic of immature neurons which is lost during development (Schwab and Bartholdi, 1996; Dusart et al., 2005). Rodent Purkinje cells progressively lose their ability to regenerate their axons during the first postnatal week: axotomy in the early postnatal period is followed by axonal regeneration, but this capacity for regeneration is absent by the second postnatal week (Dusart et al., 1997; Gianola and Rossi, 2001; Ghoumari et al., 2002). Thus, Purkinje cells, like the vast majority of CNS neurons, lose their ability to regenerate their cut axons at the end of the first postnatal week.

A ROLE FOR THYROID HORMONE IN THESE IMPORTANT TRANSITION PROCESSES?

EFFECT OF THYROID HORMONE ON PURKINJE CELL DIFFERENTIATION

Thyroid hormones (TH, which includes both thyroxine, the inactive precursor and 3,3′,5-triiodo-L-thyronine (T3), the active deiodinated derivative) are required for proper neurodevelopment (Oppenheimer and Schwartz, 1997; Koibuchi and Chin, 2000; Bernal, 2007). Whereas the T3 level in serum is remarkably stable in adults, it rapidly increases at birth and peaks during the second week of mouse postnatal development (Hadj-Sahraoui et al., 2000). It should be noted however that the distribution of T3 to neurons is a highly regulated process (Heuer and Visser, 2009), and that the exact T3 concentration sensed by Purkinje cells is unknown (Quignodon et al., 2004). In chicken embryos, it has been proposed that Purkinje cells first gain the ability to convert thyroxine into T3, increasing local signaling at early stages, and only later express the type 3 deiodinase which catabolizes TH (Verhoelst et al., 2002, 2005). Whether rodent Purkinje cells are also able to metabolize thyroxine and T3 is unknown. In rodents, T3 deficiency results in a number of histological alterations, mainly visible in cerebellum. Purkinje cell alignment is affected, their dendritic arborisations are drastically reduced, and they have fewer synapses. This cellular phenotype can be rescued only if the TH level is restored at an early stage.

T3 acts directly on gene transcription by binding to nuclear receptors (mainly TRα1 and TRβ1), which are both present in Purkinje cells. TRα1 and TRβ1 not only activate transcription upon T3 binding, but also repress gene expression in the absence of ligand. This explains why knock-out mice usually display a mild phenotype, while major neurological disorders result from point mutations making one isoform dominant negative (Flamant et al., 2002; Morte et al., 2002). The genes activated by ligand-bound TR in Purkinje cells remain completely unknown, but some reports have described changes in Purkinje cell gene expression in hypothyroid animals. For example, *Pcp2* (L7) expression is sensitive to TH deficiency, but its down-regulation is only delayed, and reaches normal levels at a later stage, as is the case for several other markers (Strait et al., 1992). This observation is consistent with the possibility that T3 only sets the timing of Purkinje cell differentiation. In line with this theory, the elimination of multiple climbing fiber innervation of Purkinje cells occurs 2–3 days later in hypothyroid animals (Crepel et al., 1981). More recently, T3 treatment in organotypic cultures was found to accelerate the progression of the early steps of Purkinje cell dendritic differentiation (Boukhtouche et al., 2010).

Although this has not been addressed in great detail, it appears that the respective abundance of both T3 receptors changes in Purkinje cells during rodent postnatal development. TRα1 appears to be expressed at birth, and then TRβ1 expression gradually increases, becoming progressively predominant after

several weeks (Mellstrom et al., 1991; Bradley et al., 1992; Wallis et al., 2010). Expressing a dominant-negative mutation of either TRα1 (Quignodon et al., 2007; Fauquier et al., 2011) or TRβ1 (Hashimoto et al., 2001; Portella et al., 2010) is sufficient to affect Purkinje cell differentiation in mice. As the two phenotypes are not identical, a cross-repression between the two receptors appears to be unlikely. The sequential expression of TRα1 and TRβ1 might be a more plausible explanation: in that case, one should be able to demonstrate that Purkinje cell differentiation is impaired at an earlier stage in TRα1 mutant. Interestingly, primary cultures showed that *in vitro* morphological changes promoted by T3 are dependent on TRα1, not TRβ1 (Heuer and Mason, 2003). In humans, many germline mutations have been reported for TRβ1 which lead to a complex syndrome without obvious cerebellar disorders, known as resistance to thyroid hormone (Weiss and Refetoff, 2000). The first case of a young patient with a TRα1 mutation was recently reported (Bochukova et al., 2012). The child's deficit was consistent with those seen in congenital hypothyroidism.

CELL AUTONOMOUS AND NON-AUTONOMOUS EFFECTS

T3 also exerts an influence on other cerebellar neuronal and glial cell populations, and can thus influence Purkinje cells indirectly. For example, Bergmann glia promote synapse formation between Purkinje cells and GABAergic interneurons (Ango et al., 2008). As T3 deficiency affects the differentiation of both Bergmann glia (Manzano et al., 2007a) and GABAergic interneurons (Manzano et al., 2007b), it has the potential to explain the observed reduction in synaptogenesis (Nicholson and Altman, 1972; Fauquier et al., 2011). T3 deficiency also impairs the production of neurotrophins by granular neurons, which stimulate Purkinje cell differentiation (Neveu and Arenas, 1996; Koibuchi et al., 2001). As the production of several other neurotrophins and growth factors by Purkinje cells changes as they mature, this creates a situation of interdependence that is very difficult to unravel. Finally, oligodendrocyte precursor differentiation, at least *in vitro*, is strictly dependent on T3 (Ahlgren et al., 1997; Durand and Raff, 2000). Myelin formation is retarded by T3 deficiency and accelerated by T3 excess (Ibarrola and Rodriguez-Pena, 1997; Marta et al., 1998; Billon et al., 2002). The temporal control exerted by T3 on the differentiation of both Purkinje cells and oligodendrocytes provides a simple hypothetical mechanism to ensure that myelination takes place soon after Purkinje axon outgrowth. Cre/loxP technology was used to address whether T3 activates early oligodendrocytes differentiation and myelin formation in a cell-autonomous manner or not. Whereas expression the dominant negative TRα1 mutation only in oligodendrocytes precursors had no visible effect on their differentiation, a delay was observed when the mutation was expressed in GABAergic neurons before P8, or when it was expressed in the astrocyte lineage (Picou et al., 2012). The ability of T3 to promote the secretion of several factors at early postnatal stage is thus likely to be determinant in the control exerted on the timing of oligodendrocytes differentiation. In that respect, T3 deficiency could be regarded mainly as a desynchronization of interdependent differentiation processes, whose consequences become rapidly irreversible.

CONCLUSIONS—PERSPECTIVES

At the end of the first postnatal week, rodent Purkinje cells undergo profound morphological and electrophysiological changes. They also deeply modify their contacts with their other cellular partners. From a cellular point of view, since these transitions occur when TH levels in serum increase (Hadj-Sahraoui et al., 2000), it is tempting to propose that Purkinje cells undergo a metamorphosis: these transitions are reminiscent of amphibian metamorphosis (Tata, 2006) and suggest an interesting analogy (Kress et al., 2009). The recent concern that some chemicals might have neurotoxic effects due to interference with T3 signaling should bring new attention to this process (Ibhazehiebo et al., 2011). Indeed, 1,2,5,6,9,10-αHexabromocyclododecane (HBCD) impairs thyroid hormone induced dendrite arborization of Purkinje cells (Ibhazehiebo et al., 2011).

With our present knowledge, it is very difficult, however, to decipher whether all the processes occurring at this transition period are only concomitant or are truly related. Furthermore, these processes could also be completely independent of TH. It is not known, for example, whether or not axon regeneration or the factors that contribute to chloride concentration within Purkinje cells are driven by TH. During this transition period, it is important to remember that Purkinje cells synthesize other hormones such as progesterone and estradiol (Tsutsui, 2008). Both progesterone and estradiol promote dendritic growth, spinogenesis, and synaptogenesis via their nuclear receptors in developing Purkinje cells (Tsutsui, 2008). Indeed, a large fraction of the 50 members of nuclear receptor family are expressed in Purkinje cells (Qin et al., 2007) suggesting that several of the small ligand molecules that bind these transcription factors are required locally. We have already described the effect of TRα, TRβ, and RORα mutations on Purkinje cells, but rev-erbα and COUP-TFII also affect either the survival or the development of Purkinje cells (Chomez et al., 2000; Kim et al., 2009). Furthermore, T3 action on early Purkinje cell dendritic differentiation requires the presence of functional RORα (Boukhtouche et al., 2010). This raises interesting questions concerning interactions between the TRα and RORα signaling pathways (Qiu et al., 2009). Furthermore, the role of RORα in later stages of development is unknown.

Finally, the direct target genes of all these nuclear receptors in Purkinje cells, and how these target genes are connected to build or maintain a functioning Purkinje cells, are currently unknown. A major innovation is the systematic development of models with somatic mutations, mainly based on CRE/loxP technology, which allow the analysis of cell autonomous consequences of these mutations (Winter et al., 2009; Fauquier et al., 2011). It will greatly improve our understanding of transcription factors, like RORα and TRα1, which are already well known to be required for Purkinje cell maturation and/or survival but are expressed in many other cell types as well. In such mutant mice, combining Purkinje cell sorting, sequence-based transcriptome analysis and electrophysiology should lead to a better characterization of the Purkinje cell status and pave the way to a deeper understanding of the molecular mechanisms at work. Whether the molecular mechanisms

governing Purkinje cell differentiation and maturation will be transposable to other cell types is difficult to predict. Whatever the extent of their originality, it is likely that this neuron will continue to occupy the minds of many researchers in the future and bring new important results for developmental neurobiology.

Another open question remains: what could be the role of such a metamorphosis in the rodents? In precocial birds (such as chicken), thyroid function is already well developed during the latter part of incubation and hatchings exhibit relatively mature sensory and locomotor capabilities: these birds are able to walk just after hatching. In contrast in altricial birds (such as dove), thyroid function shows little maturation until after hatch as also is the case for sensory, and motor functions: these birds remains in the nest for a while after hatching (McNabb, 2006). Interestingly, in contrast to rodents, the increase of TH circulation occurs before birth in sheep, an animal mature enough at birth to walk (Fisher et al., 1994). It remains to demonstrate which components of the metamorphosis of the developing cerebellar microcircuit are indeed triggered by thyroid hormone. However, it is tempting to speculate that in cerebellum as well as in other brain parts the burst availability of high levels of T3 might trigger a general process allowing the animal to switch from a developmental program to a mature one adapted to its final environment.

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Pattern formation during development of the embryonic cerebellum

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The patterning of the embryonic cerebellum is vital to establish the elaborate zone and stripe architecture of the adult. This review considers early stages in cerebellar Purkinje cell patterning, from the organization of the ventricular zone to the development of Purkinje cell clusters—the precursors of the adult stripes.

Keywords: Purkinje cell, ventricular zone, zebrin, EBF2, neurogenin, stripe

INTRODUCTION—PATTERNING OF THE ADULT CEREBELLUM

The adult mouse cerebellum houses a complex topographical map (e.g., **Figure 1**). The map probably involves all cell types in the cerebellar cortex (e.g., Purkinje cells—Hawkes et al., 1985; granule cells—Hawkes and Turner, 1994; Hawkes et al., 1998; Golgi cells—Sillitoe et al., 2008; basket cells—Demilly et al., 2011; glia—Scott, 1963): this review is focused on the Purkinje cells. The mapping of Purkinje cell antigens (e.g., zebrin II/aldolase c—Brochu et al., 1990; phospholipase (PL)Cβ4—Sarna et al., 2006; heat shock protein (HSP)25—Armstrong et al., 2000; CART—Reeber and Sillitoe, 2011 etc.), gene transcripts (reviewed in Sillitoe and Joyner, 2007) and transgenes (e.g., L7/pcp2-*lacZ*—Vandaele et al., 1991; Oberdick et al., 1993; OMP-*lacZ*—Nunzi et al., 1999; IP3R1-*nls-lacZ*—Furutama et al., 2010 etc.), has revealed multiple Purkinje cell subtypes. Each subtype has a characteristic distribution, but it seems plausible that these are all reflections of a common underlying architecture (Apps and Hawkes, 2009). First, the cerebellar cortex is divided from anterior to posterior into transverse zones: the anterior zone (AZ: ∼lobules I–V), the central zone (CZ: ∼lobules VI–VII; possibly further subdivided—Marzban et al., 2008), the posterior zone (PZ: ∼lobules VIII-dorsal IX) and the nodular zone (NZ: ∼lobules IX ventral and X: Ozol et al., 1999; Sillitoe and Hawkes, 2002). Next, each transverse zone is divided mediolaterally into parasagittal stripes. The most broadly studied marker of adult stripes is the Purkinje cell antigen zebrin II/aldolase C (e.g., Brochu et al., 1990; Ahn et al., 1994). The opposite pattern is revealed by other markers, for example PLCβ4 (Sarna et al., 2006) and EBF2 (Croci et al., 2006; Chung et al., 2008). The zone-and-stripe pattern is highly reproducible between individuals and conserved across mammals and birds: zebrin II is expressed by many vertebrates (e.g., fish—Lannoo et al., 1991a,b; Meek et al., 1992), as is EBF2 (Malgaretti et al., 1997; Bally-Cuif et al., 1998; Dubois and Vincent, 2001), but arrays of stripes are only seen in birds (e.g., pigeon—Pakan et al., 2007; chicken—Marzban et al., 2010) and mammals (reviewed in Sillitoe et al., 2005; Marzban and Hawkes, 2011). Many molecular markers are co-localized with either the zebrin II+ or zebrin II– Purkinje cells (e.g., PLCβ3—Sarna et al., 2006; sphingosine kinase 1a—Terada et al., 2004 etc.). However, this is not the extent of the stripe compartmentation—other markers reveal subdivisions within stripes, subsets of stripes within the zebrin II+/– sets, and stripes in the CZ and NZ (e.g., P-path—Leclerc et al., 1992; heat shock protein (HSP)25—Armstrong et al., 2000; human natural killer cell antigen 1 (HNK1)—Eisenman and Hawkes, 1993; Marzban et al., 2004). In sum, the adult cerebellar cortex is highly reproducibly subdivided into several hundred distinct modules with >10 distinct Purkinje cell phenotypes (e.g., reviewed in Hawkes and Gravel, 1991; Hawkes, 1997; Apps and Hawkes, 2009). In the mouse, a typical stripe/module comprises fewer than a thousand Purkinje cells.

Zones and stripes are important because cerebellar patterning influences all aspects of cerebellar organization and function. Here is not the place to expound this at length, but simply to note

FIGURE 1 | Zones and stripes in the adult mouse cerebellum. Anti-zebrin II staining of a whole mount, adult mouse cerebellum reveals an elaborate pattern of Purkinje cell stripes (for method and more details—see Sillitoe and Hawkes, 2002).

that the Purkinje cell map serves as a scaffold around which many other cerebellar structures are organized:

1. *Afferent projections* use Purkinje cells to target their terminal fields.
2. *Interneurons* are restricted at stripe boundaries and are thought to use Purkinje cell cues to establish their own topography.
3. *Functional boundaries* align with stripe boundaries (e.g., Chockkan and Hawkes, 1994; Chen et al., 1996; Hallem et al., 1999; Apps and Garwicz, 2005; Wadiche and Jahr, 2005).
4. Specific target zones in the cerebellar and vestibular nuclei receive topographically ordered projections from stripes in the cerebellar cortex (e.g., Hawkes and Leclerc, 1986; Chung et al., 2009a; Sugihara, 2011).
5. *Cerebellar mutant phenotypes* are frequently restricted at zone or stripe expression boundaries (e.g., Eisenman, 2000; Beirebach et al., 2001).
6. *Purkinje cell death* due to mutation or insult is typically restricted to parasagittal stripes (e.g., reviewed in Sarna and Hawkes, 2003).

Thus, Purkinje cell stripes lie at the heart of cerebellar structure, function, and pathology. How does this remarkable pattern develop? Where do Purkinje cell subtypes come from? How do they end up in stripes?

Cerebellar pattern formation is conventionally divided into four broad stages (the timings refer to the mouse cerebellum: **Figure 2**):

1. The formation of the cerebellar ventricular zone (∼E7–E10 in mouse);
2. Purkinje cell birth in the ventricular zone and Purkinje cell subtype specification (∼E10–E13);
3. Purkinje cell migration from the SVZ and reorganization into a stereotyped embryonic cluster array (∼E14–E17); and
4. Purkinje cell cluster dispersal and refinement to form the adult stripes (∼E18–P20).

This review is focused on stages 2 and 3—Purkinje cell subtype specification and the early stages of pattern formation. We also focus this review on patterning of Purkinje cells—less is known of the development of patterns in either granule cells or inhibitory interneurons although many of these are thought to be secondary to the patterning of the Purkinje cells (e.g., Sotelo and Chédotal, 2005; Sillitoe et al., 2008, 2010; Chung et al., 2009a,b).

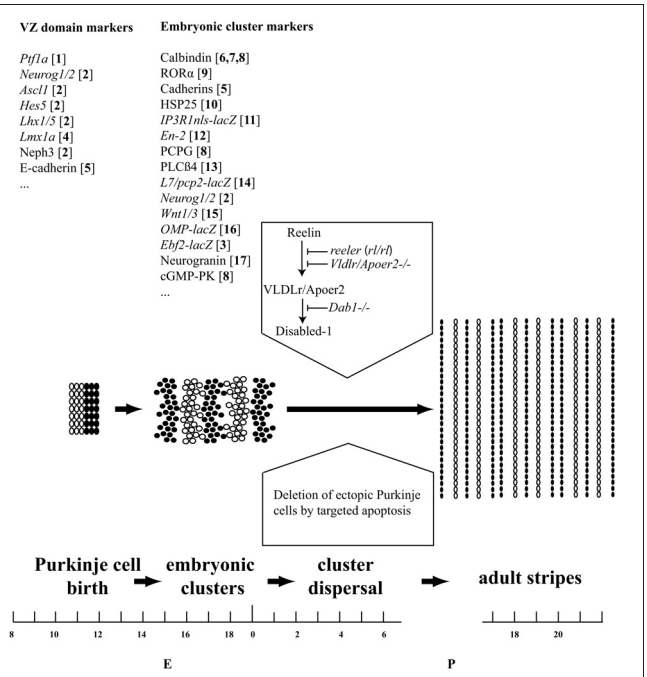

FIGURE 2 | Pattern formation in the mouse cerebellum. Cerebellar pattern formation is divided here into three broad stages: Purkinje cell birth in the ventricular zone and Purkinje cell subtype specification (∼E10–E14); Purkinje cell reorganization into a stereotyped embryonic cluster array (∼E14–E18); and cluster dispersal and refinement to form the adult stripes (∼E18–P20). Examples of markers that reveal expression domains in the VZ and early Purkinje cell clusters are listed above. References: [**1**] Hoshino et al. (2005); [**2**] Zordan et al. (2008); [**3**] Croci et al. (2006); [**4**] Chizhikov and Millen (2003); [**5**] Redies et al. (2011); [**6**] Tano et al. (1992); [**7**] Wassef et al. (1990); [**8**] Wassef et al. (1985); [**9**] Sillitoe et al. (2009); [**10**] Armstrong et al. (2001); [**11**] Furutama et al. (2010); [**12**] Millen et al. (1995); [**13**] Marzban et al. (2007); [**14**] Ozol et al. (1999); [**15**] Bally-Cuif et al. (1992); [**16**] Nunzi et al. (1999); [**17**] Larouche et al. (2006). Substantial Purkinje cell death occurs by apoptosis in the first postnatal week, and we hypothesize that this may play a role in refining the cerebellar map. The transformation of Purkinje cell clusters into stripes is triggered by Reelin binding to a Very Low Density Lipoprotein receptor/Apolipoprotein receptor 2 complex (Vldlr/Apoer2), which leads to tyrosine phosphorylation of the docking protein Dab1. These events are blocked by mutations of Reelin production (*reeler*), the Reelin receptors (*Apoer2/Vldlr* double null), or downstream signals (*Dab1–/–*).

THE STRUCTURE OF THE VENTRICULAR ZONE

The cerebellar primordium arises from the rostral metencephalon between E8.5–E9.5 (e.g., Wassef and Joyner, 1997; Sillitoe and Joyner, 2007). It houses two distinct germinal matrices, the dorsal rhombic lip and the ventral ventricular zone (VZ) of the fourth ventricle, which generate neuronal precursors fated to adopt GABAergic and glutamatergic phenotypes respectively (**Figure 3**). The earliest stage of cerebellar development depends on fibroblast growth factor 8 (FGF8) secreted by the isthmic

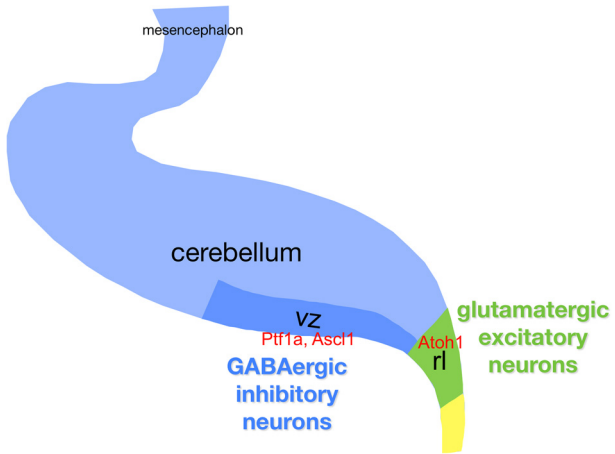

FIGURE 3 | Schematic of the cerebellar primordium with respect to neurogenesis. Gene names are in red boldface. Abbreviations: ctz, cortical transitory zone; egl, external granule layer; ntz, nuclear transitory zone; rl, upper rhombic lip; rp, roof plate; vz, ventricular zone.

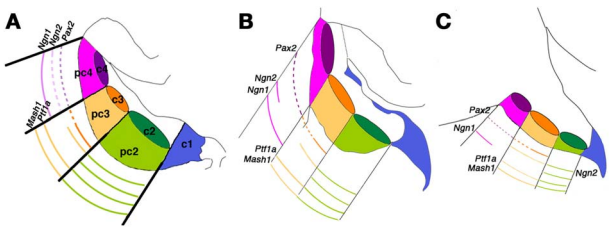

FIGURE 4 | Patterning the cerebellar ventricular zone. An integrated molecular map of embryonic day (E) 12.5 cerebellar primordium populations. This model attempts to integrate the results of Chizhikov et al. (2006) with those of Zordan et al. (2008) to provide a three-dimensional view of gene expression. **(A–C)** Representative sagittal sections spanning the mediolateral axis of the embryonic cerebellum at E12.5: **(A)** medial; **(B)** intermediate; **(C)** lateral. All sketches contain a representation of the three mitotic progenitor domains (pc2–4) and post-mitotic precursor domains (c2–4), in addition to the rhombic lip (c1). Solid lines indicate robust expression levels; dashed lines indicate lower transcript levels. Adapted from Zordan et al. (2008).

organizer (Crossley et al., 1996; Joyner, 1996; Liu et al., 1999; Martinez et al., 1999) at the midbrain-hindbrain boundary. Mutant mice with reduced *Fgf8* expression have defective cerebellar development, and ectopic FGF8 expression leads to ectopic cerebellar tissues (reviewed in Nakamura et al., 2008). FGF8 secretion initiates the expression of multiple region-specific transcription factors, including EN1/2, PAX2/5/8, OTX2, and GBX2. In addition, FGF8 initiates *Wnt1* expression at the midbrain-hindbrain boundary, and FGF8 and WNT1 form a positive feedback loop, which also involves EN2 and PAX2 (Thomas and Capecchi, 1990; McMahon et al., 1992; Bally-Cuif et al., 1992; Millen et al., 1995; Martinez et al., 1999; Simeone, 2000), and together act as organizers to pattern the tissues around the midbrain-hindbrain boundary.

Although the cerebellum contains a relatively small variety of neurons, the molecular machinery governing neuronal generation and/or subtype specification is still poorly understood. Genetic fate mapping shows that a *Ptf1a*- (pancreas transcription factor 1a—which encodes a bHLH transcription factor) expressing domain in the VZ gives rise to all Purkinje cells (Hoshino et al., 2005; Hoshino, 2006). In 2005, the characterization of a novel mutant mouse, *cerebelless*, which lacks the entire cerebellar cortex but survives into adulthood, was reported (Hoshino et al., 2005). The analysis of the phenotype, and the characterization of the underlying gene mutation, clarified that PTF1A is required for generating all cerebellar GABAergic compartment. ATOH1 and PTF1A participate in regionalizing the cerebellar neuroepithelium, and define two distinct areas, the VZ (*Ptf1a*) and the upper rhombic lip (*Atoh1*), which generate GABAergic and glutamatergic neurons, respectively (Hoshino et al., 2005; Pascual et al., 2007). In regard to GABAergic progenitors Purkinje cells are distinguished from interneurons by differential expression of E-cadherin in cycling progenitors (Mizuhara et al., 2010) and of the transcriptional corepressor Corl2 (Minaki et al., 2008) in post-mitotic precursors, while two other Ptf1a targets (Nephrin and Neph3) (Nishida et al., 2010) are expressed by all GABA progenitors. Moreover, the expression domains of three proneural genes (*Ascl1*, *Neurog1*, and *Neurog2*) overlap with that of *Ptf1a* in the VZ.

While many studies have investigated the roles played by ATOH1 in establishing the cerebellar glutamatergic lineage, fewer studies have explored GABAergic precursors born in the cerebellar VZ. In 2008, Zordan et al. published a systematic descriptive analysis of proneural gene expression at early stages of mouse cerebellar development (**Figure 4**). This established that at the onset of cerebellar neurogenesis (∼E11), the *Ascl1* transcript becomes detectable in the VZ and presumptive NTZ. A similar distribution is observed at later stages, with the *Ascl1* transcript occupying the entire thickness of the *Ptf1a*+ VZ all the way to its apical (ventricular) margin. Accordingly, the territories occupied by *Ascl1* and *Atoh1* are clearly complementary. *Ascl1* remains confined to the VZ until E13.5. An additional study by Johnson and coworkers (Kim et al., 2011) described genetic fate mapping studies done by using two transgenic *Ascl1*-Cre lines, one of which expressed a tamoxifen-inducible Cre recombinase, CreERTM (Helms et al., 2005; Battiste et al., 2007) and two Cre-inducible reporter lines (Soriano, 1999; Srinivas et al., 2001). The evidence produced in this elegant lineage analysis study is in full agreement with Zordan et al. In particular, *Ascl1*+ progenitors are initially (E12.5) restricted to the cerebellar VZ and excluded both from the post-mitotic cerebellar transitory zone and from the rhombic lip migratory stream. The *Ascl1*+ and *Atoh1*+ progenitor domains are mutually exclusive, whereas a high degree of overlap exists between *Ptf1a*+ and *Ascl1*+ progenitors, suggesting that *Ascl1* labels GABAergic neuronal progenitors. However, by E17.5 *Ascl1*+ progenitors are no longer confined to the VZ but are also found scattered throughout the cerebellar primordium.

Finally, a study by Wassef and co-workers (Grimaldi et al., 2009) further refined the analysis of the role of *Ascl1* in cerebellar neurogenesis, incorporating the effects of *Ascl1* gene disruption and overexpression. They established that *Ascl1*+ progenitors progressively delaminate out of the VZ to settle first in the

prospective white matter, and then in the cerebellar cortex. By studying an *Ascl1*-GFP transgenic mouse, they demonstrated that *Ascl1*+ progenitors give rise to PAX2+ interneurons and OLIG2+ oligodendrocyte precursors, while glutamatergic neurons, astrocytes and Bergmann glial cells did not express GFP. In contrast, the loss of *Ascl1* led to a dramatic reduction of PAX2+ and OLIG2+ precursors. No change was found in Purkinje cell development.

Finally, a gain-of-function approach by using *in vivo* electroporation of a GFP plasmid at E14.5, concluded that most *Ascl1*+ oligodendrocytes do not originate from the cerebellar VZ. In addition, an *Ascl1* plasmid electroporated into the cerebellar VZ led to an increased number of PAX2+ interneurons, fewer OLIG2+ oligodendrocyte precursors, and the complete absence of astroglia. This suggests that *Ascl1* overexpression pushes progenitors toward a (PAX2+) interneuron fate and suppresses the astrocytic fate.

Taken together, evidence suggests that *Ascl1* contributes to GABAergic interneuron and cerebellar nuclear neuron generation, and to Purkinje cell development. However, it is not required for Purkinje cell specification.

POSSIBLE ROLES FOR NEUROGENINS IN THE DEVELOPMENT OF CEREBELLAR GABAergic NEURONS

Neurog1 and *Neurog2* are expressed in the *Ptf1a*+ ventricular neuroepithelium. As shown by Zordan et al. (2008), the *Neurog2* transcript is first observed around E11 in cerebellar nuclear neuron progenitors of the cerebellar primordium, whereas *Neurog1* appears 1 day later, in a rostral region located between the isthmic organizer, labeled by Fgf8, and the territory marked by *Ascl1*. At E12.5, both *Neurog1* (see also Salsano et al., 2007) and *Neurog2* are present in the VZ but with a few differences in distribution: in the anterior cerebellum, *Neurog1* is expressed at high levels in a region close to the midline, whereas *Neurog2* is restricted to the lateral VZ. In posterior territories, the expression patterns overlap completely. *Neurog1* and *Neurog2* are adjacent to, and partially overlap with, post-mitotic domains labeled by *Lhx1* and *Lhx5*, two genes that control Purkinje cell differentiation (Zhao et al., 2007). This suggests that *Neurog1* and *Neurog2* are expressed in progenitors that are undertaking the last cycle of cell division to become post-mitotic Purkinje cell precursors. At E13.5 the differential anterior boundaries of *Neurog1* and *Neurog2* are maintained, although the transcript levels of both genes are down-regulated. The authors conclude that *Neurog1* and *Neurog2* are mainly expressed in the cerebellar germinal epithelium that gives rise to GABAergic progenitors, while they are completely absent from the rhombic lip, the source of all glutamatergic cerebellar progenitors. Moreover, their expression patterns are similar but not totally overlapping, suggesting that these two closely related genes may contribute to the diversity of cerebellar GABAergic neurons and, possibly, Purkinje cell subtypes.

Neurog1 IS EXPRESSED IN CEREBELLAR GABAergic INTERNEURON PROGENITORS

In 2009, Doughty and coworkers published a lineage analysis study that described the mature cerebellar neurons deriving from *Neurog1*+ cell fates in the developing mouse cerebellum (Lundell et al., 2009). They confirmed the findings of Zordan et al. (2008) and extended the analysis to late embryonic and postnatal cerebellar development. At E14–E20, *Neurog1* is present in Ptf1a+ neurons, but it is excluded from the upper rhombic lip and external granular layer. Moreover, at P7, it co-localizes with Ptf1a and BrdU in the deep white matter. This suggests that *Neurog1* is expressed in early GABAergic interneuron precursors that, shortly after birth, migrate from the white matter to reach their final destination in the cortex. By using two artificial chromosome (BAC)-reporter mice they analyzed short-term and long-term Neurog1+ cell fates. *Neurog1* is expressed in PAX2+ interneuron progenitors but it does not contribute to the GABAergic neuron lineage in the cerebellar nuclei (Vue et al., 2007). Surprisingly, they did not reveal any fluorescence in Purkinje cells. Furthermore, the authors bred *Neurog1-Cre* transgenic mice into the double reporter Z/EG line (Novak et al., 2000). Z/EG mice express a LacZ cassette under control of a CMV enhancer/chicken actin promoter (pCAGGS). In the presence of a Cre recombinase the *lacZ* cassette is excised, leading to the activation of the downstream *EGFP* gene. By using this approach, they revealed scattered GFP+ Purkinje cell neurons, mostly in the hemispheres. Surprisingly, this strategy failed to tag GABAergic interneurons, perhaps due to epigenetic/positional silencing of the reporter transgene or to low-level expression of the *Neurog1-Cre* transgene. A recent study confirmed the notion that, in the cerebellar primordium, the *Neurog1*+ lineage contributes to the Purkinje cell pool (Kim et al., 2011).

In summary, *Neurog1* is expressed in progenitors giving rise to GABAergic interneurons of the cerebellar cortex and at least some Purkinje cells. However, it does not seem to contribute to the development of neurons of the cerebellar nuclei. While both neurogenin genes are expressed in the cerebellar VZ in presumptive GABAergic neurons, nothing can be inferred to date as regards their function(s). Do they affect cell type or subtype specification, or neuronal vs. glial commitment? And in either case, do they act redundantly with each other or with *Ascl1?* There seems to be some degree of selectivity, in that *Neurog1* is expressed in only a share of GABAergic progenitors and, as predicted by Zordan et al. (2008), the broader expression domain exhibited by *Neurog2*, a direct PTF1A target gene (Henke et al., 2009), suggests that it may play a unique role in the development of GABAergic cerebellar nuclear neurons. No mechanism has been identified but a recent study of gene expression in the cerebellar primordium of E11.5 *Neurog1* null mice suggests that Neurog1 and Pax6 may interact functionally in the activation of downstream targets (Dalgard et al., 2011).

PURKINJE CELL BIRTH AND THE FORMATION OF THE FIRST LAMINAR PHASE

Purkinje cells undergo terminal mitosis in the VZ between E10–E13 in the mouse (Miale and Sidman, 1961; Hashimoto and Mikoshiba, 2003; Namba et al., 2011: **Figure 2**). Birthdating studies, using incorporation of either adenovirus (Hashimoto and Mikoshiba, 2003) or bromodeoxyuridine (e.g., chick—Karam et al., 2000; mouse—Larouche and Hawkes, 2006), reveal a direct correlation between the birthdate of a Purkinje cell and its final mediolateral location, suggesting that Purkinje cells acquire

positional information at or shortly after their terminal differentiation in the VZ. It is not known whether positional information and phenotype are specified at the same time. Post-mitotic Purkinje cells migrate dorsally out of the VZ, in part along radial glia processes (e.g., Morales and Hatten, 2006), and stack in the cerebellar anlage with the earliest-born Purkinje cells located most dorsally.

SPECIFICATION OF PURKINJE CELL SUBTYPES

In many areas of the CNS, the development of patterning is driven by neuronal activity (e.g., the retinotectal system—reviewed in Ruthazer and Cline, 2004), In contrast, Purkinje cell phenotype specification and stripe formation seem to be activity-independent. Experiments both *in vivo* and *in vitro* suggest that the zebrin phenotype is specified early in development and is not influenced by subsequent interactions with cerebellar afferents, cerebellar neurons or glia (Leclerc et al., 1988; Wassef et al., 1990; Seil et al., 1995). For example, Purkinje cells in P0 cerebellar slice cultures express both zebrin+/− phenotypes, and blocking neuronal activity or depleting granule cells and glia did not change this (Seil et al., 1995). Next, deafferentation of the neonatal or adult cerebellum does not alter the fundamental zone and stripe architecture (zebrin I—Leclerc et al., 1988; HSP25—Armstrong et al., 2001). Similarly, cerebellar anlagen dissected from embryos at E12–E15 (prior to any contact with afferents—Paradies and Eisenman, 1993; Grishkat and Eisenman, 1995) and transplanted into either the anterior chamber of the eye or the neocortex of adult hosts (Wassef et al., 1990) had zebrin II+/− Purkinje cells in the mature grafts. Furthermore, zebrin+ Purkinje cells are more numerous in cultures of posterior cerebellum than anterior cerebellum, consistent with the expression pattern seen *in vivo* (Leclerc et al., 1988; Hawkes, unpublished data). Finally, several experiments have suggested a correlation between the time when a Purkinje cell is born and its final mediolateral position in the mature cerebellum (Hashimoto and Mikoshiba, 2003; Larouche et al., 2006) suggesting that Purkinje cells' adult phenotypes are specified shortly after their birth in the VZ.

What is known of Purkinje cell subtype specification? EBF2 is one of four members of a family of helix-loop-helix transcription factors highly conserved in evolution (reviewed in Dubois and Vincent, 2001; Liberg et al., 2002) that couple cell cycle exit to the onset of neuronal differentiation and migration (Garcia-Dominguez et al., 2003). Three of these (*Ebf1-Ebf3*) encode transcriptional activators expressed in cerebellar development (**Figure 2**, Croci et al., 2006). While no cerebellar defects have been described in *Ebf1* or *Ebf3* mutants, *Ebf2* null mice feature a small cerebellum and apoptotic cell death of migrating and post-migratory Purkinje neurons. Of the Purkinje cells that survive, a major fraction is transdifferentiated into the zebrin II+ phenotype (Croci et al., 2006). An analysis of molecular markers of Purkinje cell subtypes revealed that EBF2 acts specifically to repress the zebrin II+ subtype, rather than to maintain the zebrin II– one (Chung et al., 2008). Interestingly, unlike EBF3, EBF2 is sensitive to Notch-mediated repression in *Xenopus* neurulae (Pozzoli et al., 2001), both at the transcriptional and at the functional level, suggesting that Notch signaling may affect the determination of Purkinje cell subtypes by modulating EBF2, for instance by switching off *Ebf2* expression in the early born Purkinje cell population. Consistent with this hypothesis, genetic tagging of EBF2+ cells by using an *Ebf2-Cre* transgene reveals that all Purkinje cells are initially *Ebf2*+ (Consalez, unpublished). Conditional overexpression experiments are now required to elucidate the crucial stages at which EBF2 affects Purkinje cell subtype specification in pre- and postnatal development.

THE MIGRATION OF PURKINJE CELLS TO FORM EMBRYONIC CLUSTERS

Post-mitotic Purkinje cells migrate from the VZ and stack in a layer with the earliest-born located dorsally (and becoming zebrin II+) and the youngest ventrally (and becoming EBF2+: **Figure 2**). Subsequently the layer undergoes a quite complicated reorganization (Miyata et al., 2010), possibly involving cell-signaling molecules including cadherins (e.g., Neudert and Redies, 2008; Redies et al., 2011) and ephrins (e.g., Karam et al., 2000; Sentürk et al., 2011), to yield a stereotyped array of embryonic Purkinje cell clusters with multiple molecular phenotypes. Grafts of dissociated Purkinje cells also organize into discrete zebrin+/− compartments, pointing to cell-cell adhesion molecules as possible organizers (Rouse and Sotelo, 1990). Each Purkinje cell cluster is separated from its neighbors by narrow gaps ("raphes"), later filled by migrating granule cells. In general terms, expression data show up to 10 embryonic clusters, arrayed symmetrically from medial to lateral on each side of the midline.

CLUSTER ARCHITECTURE IN THE EMBRYONIC CEREBELLUM

The reorganization of the early lamina results in a highly reproducible array of Purkinje cell clusters that can be distinguished through the differential expression of numerous molecules. The expression profiles are of three kinds. First, there are molecules that are selectively expressed at some time during embryogenesis but subsequently disappear and are not expressed in the adult (e.g., neurogranin—Larouche et al., 2006). Secondly, some molecules are selectively expressed during embryogenesis but are expressed by all Purkinje cells in the adult (e.g., calbindin—Wassef et al., 1985). Thirdly, a few molecules are selectively expressed by Purkinje cell subsets both in the embryo and the adult (e.g., PLCβ4—Marzban et al., 2007); and finally, in some cases expression reveals one pattern in the neonate and a different one in the adult (e.g., HSP25—Armstrong et al., 2001).

Examples of embryonic cluster markers include:

Calbindin (Calb1) is a major calcium binding protein that acts as a buffer to protect neurons from neurotoxicity. At around P0, calbindin expression defines three Purkinje cell clusters on each side of the midline (Wassef et al., 1985; Larouche et al., 2006). In the adult cerebellum, calbindin is expressed uniformly by all Purkinje cells

Phospholipase Cβ4 (PLCβ4) is a signal transducer. PLCβ4-immunoreactive Purkinje cells in neonatal mice reveal two, three, and four parasagittal domains in the AZ, CZ, and PZ respectively. Later, differential expression of PLCβ4 in the AZ and PZ reveal a striped pattern in the mature cerebellar cortex (Marzban et al., 2007).

Engrailed-2 (En2) is a transcription factor important for the differentiation of Purkinje cells and the adult ("late-onset") banding pattern. En2 expression, labels three distinct cluster domains at E17.5 but expression is suppressed in Purkinje cells after birth (Millen et al., 1995).

Cadherins mediate cell adhesion and play fundamental roles in the growth and development of many cells. Purkinje cell clusters express multiple members of this superfamily and some, such as cdh8, pch7, and pcdh10, are expressed differentially. For example, in the mouse cerebellar cortex at P3 cdh8-immunoreactive Purkinje cells form two parasagittal clusters each side of the midline, there are three pch7+ clusters, and a single pcdh10 cluster. In some cases, such as pcdh10, expression is maintained and the cerebellum displays a striped pattern in the adult cortex (Redies et al., 2011).

Heat shock protein 25 (HSP25) is involved in stress resistance by acting as a chaperone that binds to and stabilizes the active conformations of other proteins. At P1, the anterior lobe of the mouse cerebellum presents two distinct pairs of clusters of HSP25+ Purkinje cells arrayed symmetrically about the midline in the AZ and PZ (Armstrong et al., 2001). During later postnatal development HSP25 is transiently expressed by all Purkinje cells, until in the adult expression becomes restricted to a quite different pattern of stripes (in the CZ and NZ: Armstrong et al., 2000, 2001).

Purkinje cell protein 2-lacZ transgene (L7/pcp2-lacZ): pcp2 is a G-protein regulator that is widely expressed in Purkinje cells. Around P0, the differential expression pattern of an L7/pcp2-lacZ transgene reveals three distinct compartments in each hemicerebellum (Oberdick et al., 1993; Ozol et al., 1999). In the adult cerebellum, L7/pcp2-lacZ expression remains in stripes of Purkinje cells, especially in the AZ and PZ (Ozol et al., 1999).

Synaptotagmin IV (Syt IV) is involved in early neural differentiation including axonal growth and the formation and consolidation of synapses. At P0, Syt IV is weakly expressed in select Purkinje cell clusters. From P15 onward, all Purkinje cells are Syt IV+ (Berton et al., 1997).

Neurogranin (Nrgn) is a neural calmodulin-binding protein thought to play an important role in synaptic transmission and neuronal plasticity. At E17, Nrgn expression in the AZ and PZ reveals three and four parasagittal pairs of neurogranin expressing Purkinje cell clusters respectively (Larouche et al., 2006). These disappear in the adult.

Inositol 1,4,5-trisphosphate (IP3) receptor-lacZ transgene (IP3Rnls-lacZ): IP3R is a ligand-gated calcium channel, which is highly expressed in Purkinje cells. From E15 to P0, two clusters of Purkinje cells are selectively labeled on either side of the midline. Transgene expression continues to reveal heterogeneous Purkinje cells stripes in both the vermis and the hemisphere in the adult (Furutama et al., 2010).

Wnt7b is a signaling molecule involved in CNS development. At E18, Wnt7b expression reveals three mediolateral Purkinje cells clusters: Wnt7b expression is shut down in the adult (Hashimoto and Mikoshiba, 2003).

Olfactory marker protein-lacZ transgene (OMP-lacZ): The pattern of expression of an OMP-lacZ fusion gene (from E14.5 to P0) demonstrates three clusters on each side of the cerebellar midline. In the adult cerebellum, the pattern of transgene expression continues to reveal a striped pattern, restricted to the posterior lobe (Nunzi et al., 1999).

Early B-cell factor 2 (EBF2): Sections through the mouse cerebellum show EBF2 is expressed shortly after birth in multiple stripes and wholemount staining of adult cerebellum shows EBF2-lacZ is expressed in stripes restricted to the AZ and CZ, equivalent to the distribution of the zebrin II– Purkinje cell subset (Croci et al., 2006).

Cyclic GMP-dependent protein kinase (cGK) is implicated in multiple biological functions, including axon guidance, synaptic plasticity and learning. Transverse sections of rat cerebellum taken between E17–P3 reveal two discrete clusters of Purkinje cells that are immunoreactive for cGK. In adults, all Purkinje cell express cGK (De Camilli et al., 1984; Wassef et al., 1985).

Ephrin type-A receptor 4 (EphA4): is a tyrosine kinase receptor. Transverse sections through the mouse cerebellum at E18.5 display four distinct EphA4+ clusters of Purkinje cells (Hashimoto and Mikoshiba, 2003). In the adult cerebellum, EphA4 expression appears homogenous, except perhaps for some areas of the hemispheres (Karam et al., 2000).

PEP-19: is a developmentally regulated polypeptide that modulates calmodulin function. The expression pattern demonstrates three clusters on each side of the cerebellar midline (Herrup and Kuemerle, 1997). PEP19 expressed in all Purkinje cells of the adult rat cerebellum (Mugnaini et al., 1987).

While these, and other, markers reveal embryonic cerebellar complexity, the relationships between the various topographic maps are poorly understood. A speculative but most useful synthesis is presented in Herrup and Kuemerle (1997).

FROM CLUSTERS TO STRIPES

Purkinje cell cluster dispersal is triggered at around birth by Reelin secreted by the external granular layer (D'Arcangelo et al., 1995, 1997; Miyata et al., 1997; Tissir and Goffinet, 2003: **Figure 2**). Reelin binds two receptors on Purkinje cells—Apolipoprotein E receptor 2 (*Apoer2*) and the very low density lipoprotein receptor (*Vldlr*: Trommsdorff et al., 1999; Hiesberger et al., 1999). Binding induces receptor clustering (Strasser et al., 2004) and activates a protein kinase cascade leading to tyrosine phosphorylation of the docking protein Disabled (Dab1: Goldowitz et al., 1997; Howell et al., 1997; Sheldon et al., 1997; Gallagher et al., 1998; Rice et al., 1998). Downstream of Dab1 are multiple kinase pathways Src and Fyn tyrosine kinases (Bock and Herz, 2003; Kuo et al., 2005), cyclin-dependant kinase 5 (Ohshima and Mikoshiba, 2002 etc.). The end result is thought to be a drop in mutual Purkinje cell-Purkinje cell adhesion, thereby freeing the embryonic clusters to disperse into stripes.

When this pathway is disrupted by mutation of Reelin (*reeler*: D'Arcangelo et al., 1995, 1997), Reelin receptors (*Apoer2/Vldlr* double null: Trommsdorff et al., 1999) or the Dab1 docking protein (*Dab1–/–:* Howell et al., 1997) *all* cluster dispersal is blocked. However, in contrast to the full *reeler* phenotype with no embryonic cluster dispersal, several mutations cause a *partial*

reeler phenotype—some Purkinje cells remain as ectopic clusters in the cerebellar core while most disperse normally to form stripes (*weaver*—Armstrong and Hawkes, 2001; *rostral cerebellar malformation*—Ackerman et al., 1997; *cerebellar deficient folia*—Beirebach et al., 2001, etc.). For some of these, mutations in the human homologs are known similar cerebellar phenotypes (e.g., Reelin—Hong et al., 2000; *Vldlr*—Boycott et al., 2005).

In the embryo the cluster is ∼10 Purkinje cells deep. As the clusters disperse into adult stripes the Purkinje cells spread to form a monolayer. Because dispersal occurs primarily in the anteroposterior plane, as the lobules of the cerebellum form, the rostrocaudal length of the cerebellum increases ∼25-fold while the width of the vermis increases only ∼1.5-fold (Gallagher et al., 1998). As a result the clusters string out into long parasagittal stripes. Most adult stripe markers are first expressed during this period. A few already show more-or-less adult patterns of restriction by around P5 (e.g., PLCβ4—Marzban et al., 2007) but most—including zebrin II (Lannoo et al., 1991a,b)—are first expressed at around this time but go through a "global expression" phase in which they are expressed by all Purkinje cells (e.g., HSP25—Armstrong et al., 2001; zebrin II—Lannoo et al., 1991a; Rivkin and Herrup, 2003; OMP-lacZ—Nunzi et al., 1999) before they are selectively down-regulated and the stripe architecture matures by P20.

What is the topographical relationship between the embryonic clusters and the adult stripes? By E18, numerous Purkinje cell molecular markers show restriction to subsets of clusters. The accumulated data from expression mapping of single markers suggest the possibility of a straightforward embryonic architecture: all known early markers appear to be restricted to the same schema with no more than ∼10 clusters on each side of the midline. So why are there many more adult Purkinje cell stripes than there are embryonic clusters (several hundred stripes vs. a few dozen clusters)? One explanation is that clusters are much more complex than is generally appreciated. By this view, the elaborate adult topography arises because each "simple" embryonic cluster in fact comprises multiple sub-clusters. In some cases there may be internal partitions (e.g., a medial vs. a lateral component of a cluster, each becoming a separate stripe in the adult); in other cases, Purkinje cells of different phenotypes may be intermingled within a cluster but segregate into separate stripes as the cluster transforms into stripes. Alternatively, each embryonic cluster may be homogeneous and additional complexity introduced into the adult map because individual clusters disperse into multiple stripes of the same adult phenotype. One previous study supports "complex dispersal"—in the *weaver* mouse two clusters fail to disperse and three adult stripes are missing, all of the zebrin II+/HSP25+ phenotype (Armstrong and Hawkes, 2001). Sillitoe et al. (2009) reveal a similar story by using a pcp2-CreER-IRES-hAP transgene to tag three bilateral clusters on approximately E15 and show they yielded zebrin II+ Purkinje cells of nine adult stripes. On the other hand, in some cases several embryonic clusters merge to form a single stripe. The clearest example are the zebrin II–/PLCβ4+ stripes in the vermis of the AZ, which are seen to be subdivided into triplets by the pattern of mossy fiber innervation (Ji and Hawkes, 1994) and arise from the fusion of three perinatal PLCβ4+ clusters (Marzban et al., 2007).

ROLE OF PROGRAMMED PURKINJE CELL DEATH?

Finally, during the perinatal period it is clear that significant Purkinje cell death occurs (reviewed in Vogel, 2002: **Figure 2**). Does this play a role in the sculpting of cerebellar topography? Two complementary hypotheses can be considered. First, studies of naturally occurring cell death in the cerebellum have identified a spatial organization to Purkinje cell apoptosis ("hot spots": Jankowski et al., 2009) that correlates with stripe boundaries in the adult, and propose the interesting hypothesis that cell death may sharpen the acellular raphes between clusters. In addition, naturally occurring cell death could be an error-correction mechanism. A striking feature of adult cerebellar topography is its high reproducibility between individuals and its attendant low error rate (e.g., zebrin II+ Purkinje cells are very rarely seen in zebrin II– stripes). If stripes derive from clusters, and stripes have no errors, then either clusters have no errors (and migration from the VZ to the clusters is perfect) or errors that occur during cluster formation are subsequently eliminated. In this context it is interesting that many Purkinje cells—perhaps as many as a third—undergo cell death by apoptosis during the perinatal period (Dusart et al., 2006; Jankowski et al., 2009). This suggests the hypothesis that perinatal apoptosis might eliminate Purkinje cells that wind up in the wrong embryonic cluster (possibly via a local insulin-like growth factor 1 pathway—Croci et al., 2011; see also Jung et al., 2008). Purkinje cell ectopia is not lethal *per se*: for example, clusters that fail to disperse normally do not die [e.g., *reeler* (Goffinet, 1983; Edwards et al., 1994), *Vldlr–/–:Apoer2–/–* (Larouche et al., 2008), *Dab1–/–* (Howell et al., 1997)], and Purkinje cells located ectopically in the molecular or granular layers survive indefinitely (e.g., Rouse and Sotelo, 1990; Carletti et al., 2008). Rather, one might evoke a community effect (à la Yang et al., 2002), such that being in the wrong cluster during development leads to apoptosis.

PURKINJE CELL ARCHITECTURE AS A SCAFFOLD FOR CEREBELLAR TOPOGRAPHY

It is generally believed that the Purkinje cell architecture is the scaffolding around which many other cerebellar components are organized (e.g., reviewed in Sotelo and Wassef, 1991). For example, both climbing fiber and mossy fiber afferents terminate in the cerebellum as stripes that align with those revealed by stripe antigens (e.g., climbing fibers—Chédotal et al., 1997; Sotelo and Chédotal, 2005; mossy fibers—Sotelo and Wassef, 1991; Ji and Hawkes, 1995; Armstrong et al., 2009). In some cases, this can be very precise: for example somatostatin-immunoreactive mossy fibers terminate precisely beneath a very small subset of Purkinje cell stripes (∼2%) that constitutively express HSP25 (Armstrong et al., 2009). The alignment of the afferent and Purkinje cell (= efferent) maps is established early in cerebellar development where the earliest mossy fiber topography is seen as transient, possibly functional, contacts between mossy fibers and Purkinje cells (e.g., Mason and Gregory, 1984; Takeda and Maekawa, 1989) in specific embryonic clusters (Grishkat and Eisenman, 1995; Paradies et al., 1996). When the embryonic clusters disperse into stripes the afferents appear to move with them, thereby retaining the topographic relationship with a particular Purkinje cell subset. During postnatal development, mossy fibers move to the

granular layer but stay aligned with the Purkinje cell stripe (e.g., Arsénio Nunes and Sotelo, 1985; Ji and Hawkes, 1995). A similar mechanism seems to serve to guide cerebellar interneurons to their specific stripe locations (unipolar brush cells—Chung et al., 2009a,b; Golgi cells—Sillitoe et al., 2008) and boundaries between stripes restrict the mediolateral spread of Golgi cell dendritic arbors (Sillitoe et al., 2008).

Finally, a different, and not well-understood, process also restricts granule cell dispersal. There are several subclasses of granule cell based both on gene expression (e.g., reviewed in Hawkes and Eisenman, 1997; Ozol and Hawkes, 1997) and lineage (Hawkes et al., 1998). Transverse boundaries that separate granule cell lineages align with transverse zone boundaries identified in the Purkinje cell scaffold. How this comes about is not understood.

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An agonist–antagonist cerebellar nuclear system controlling eyelid kinematics during motor learning

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The presence of two antagonistic groups of deep cerebellar nuclei neurons has been reported as necessary for a proper dynamic control of learned motor responses. Most models of cerebellar function seem to ignore the biomechanical need for a double activation–deactivation system controlling eyelid kinematics, since most of them accept that, for closing the eyelid, only the activation of the orbicularis oculi (OO) muscle (via the red nucleus to the facial motor nucleus) is necessary, without a simultaneous deactivation of levator palpebrae motoneurons (via unknown pathways projecting to the perioculomotor area). We have analyzed the kinetic neural commands of two antagonistic types of cerebellar posterior interpositus neuron (IPn) (types A and B), the electromyographic (EMG) activity of the OO muscle, and eyelid kinematic variables in alert behaving cats during classical eyeblink conditioning, using a delay paradigm. We addressed the hypothesis that the interpositus nucleus can be considered an agonist–antagonist system controlling eyelid kinematics during motor learning. To carry out a comparative study of the kinetic–kinematic relationships, we applied timing and dispersion pattern analyses. We concluded that, in accordance with a dominant role of cerebellar circuits for the facilitation of flexor responses, type A neurons fire during active eyelid downward displacements—i.e., during the active contraction of the OO muscle. In contrast, type B neurons present a high tonic rate when the eyelids are wide open, and stop firing during any active downward displacement of the upper eyelid. From a functional point of view, it could be suggested that type B neurons play a facilitative role for the antagonistic action of the levator palpebrae muscle. From an anatomical point of view, the possibility that cerebellar nuclear type B neurons project to the perioculomotor area—i.e., more or less directly onto levator palpebrae motoneurons—is highly appealing.

Keywords: classical eyeblink conditioning, cerebellar interpositus neurons, kinetic neural commands, eyelid kinematics, motor learning, agonist–antagonist system, timing, dispersion patterns

INTRODUCTION

The deep cerebellar nuclei are the exclusive source of cerebellar output to red nucleus, thalamus, and inferior olive after integrating inhibitory inputs from cerebellar cortical Purkinje cells with excitatory inputs from spinal cord and brainstem sources. Most models of cerebellar function assume a simple neural activation system controlling eyelid kinematics during motor learning—i.e., a simple dynamic association between the firing activity of neurons in cerebellar cortical and/or interpositus nucleus and the proper performance of conditioned eyelid responses. However, the neural and biomechanical complexity of the different forms of cerebellar learning, call this assumption into question (Ito, 1984; Welsh and Harvey, 1991; Krupa et al., 1993; Llinás and Welsh, 1993; Mauk, 1997; Hesslow and Yeo, 1998; Bracha et al., 2001; Delgado-García and Gruart, 2002; Morcuende et al., 2002; Christian and Thompson, 2003; Freeman and Steinmetz, 2011). Indeed, the different functional types of cerebellar nuclei neurons (Gruart and Delgado-García, 1994; Gruart et al., 1997, 2000a; Chen and Evinger, 2006) and eyelid movements (Evinger et al., 1991; Gruart et al., 1995; Pellegrini and Evinger, 1995; Gruart et al., 2000b) increase the difficulty to understand the role of the cerebellum in motor learning.

A simple eyelid blink involves an integrated biomechanical system of inertial, elastic, and viscous elements, and three active muscular forces in a motor sequence of activation–deactivation of the orbicularis oculi (OO) and levator palpebrae superioris (LPS) muscles. In addition, in those species with a third eyelid, there is the active contraction of the retractor bulbi muscle as well as a pressure force produced by eye retraction into the ocular orbit that squeezes the Harder's gland and passively displaces the attached nictitating membrane (NM) over the cornea (Berthier and Moore, 1990; Berthier et al., 1991; Bartha and Thompson,

Abbreviations: CRs, conditioned responses; CS, conditioned stimulus; EMG, electromyography; IPn, interpositus neuron; LPS, levator palpebrae superioris; OO, orbicularis oculi; US, unconditioned stimulus.

1992a,b; Evinger and Manning, 1993; Lepora et al., 2007, 2009; Mavritsaki et al., 2007). The neural complexity of this motor sequence is also evident by the synergistic contributions of the recruited motor [RB motoneurons (Mns), OO Mns, and LPS Mns located in the accessory abducens, facial, and oculomotor nuclei, respectively] and premotor [the action of cerebellar interpositus nucleus via the red nucleus] neuronal units (McCormick and Thompson, 1984; Berthier and Moore, 1990; Gruart and Delgado-García, 1994; Pellegrini et al., 1995; Trigo et al., 1999; Morcuende et al., 2002; Delgado-García and Gruart, 2002, 2005; Chen and Evinger, 2006). In addition, some authors suggest that the globe retraction into the ocular orbit and NM extension over the cornea, as well as the angular displacement of the eyelid on the ocular globe, should be modeled as a linked non-linear system with a more-biophysically based set of equations (Huxley, 1957; Hung et al., 1977; Trigo et al., 2003; Sánchez-Campusano et al., 2003), simultaneously taking into account the relative contributions of eyelid and eye-retraction motor systems to reflex and classically conditioned blink responses, as reported in studies carried out in the rabbit (McCormick et al., 1982; Leal-Campanario et al., 2004).

Recordings of deep cerebellar nucleus neurons during the conditioned eyelid paradigm suggest that interpositus neurons (IPns) facilitate eyelid closure of conditioned and reflex blinks (Gruart et al., 2000a; Porras-García et al., 2010; Campolatarro et al., 2011; Freeman and Steinmetz, 2011). A series of careful experimental studies of eyelid conditioning in behaving cats carried out in our laboratory reveals two types of blink-related IPn—one (labeled type A) that increases its instantaneous firing frequency with the beginning of an eyelid response, and a second type (labeled type B) that decreases its instantaneous firing frequency during the performance of the eyelid responses (Gruart et al., 1997, 2000a; Delgado-García and Gruart, 2002, 2005; Jiménez-Díaz et al., 2004). Those authors interpret the type A IPns as facilitating the OO muscle during eyelid closing, and the type B IPns as promoting activation of the LPS muscle during eyelid opening. In recent studies (Sánchez-Campusano et al., 2007, 2009, 2011a,b), we have worked on integrating the experimental and analytical approaches for a better understanding of the relationships between the kinetic neural commands and the performance (kinematics) of learned eyelid responses. In those works, we have re-analyzed (with an exhaustive analytical approach) the firing activities of only type A cerebellar posterior IPns and OO Mns in alert behaving cats during classical eyeblink conditioning, using a delay paradigm.

Accordingly, we decided to investigate in detail the firing properties of type B IPns (i.e., the neurons that pause during any active downward displacement of the upper eyelid) in comparison with the firing properties of type A IPns (i.e., the neurons that exert a reinforcing-modulating action, via the red nucleus, on OO Mns during the closing of the eyelid), with the help of timing and time-dispersion pattern analyses, as well as raster and 3D representations in the time and frequency domains. Here, we present some functional evidence that the presence of two antagonistic groups of deep cerebellar nuclei neurons is necessary for a proper dynamic control (proper timing and kinetic–kinematic characterization) of eyelid movements.

MATERIALS AND METHODS

EXPERIMENTAL SUBJECTS

Experiments were carried out with eight adult cats (weighing 2.1–3.2 kg) obtained from an authorized supplier (Iffa-Credo, Arbresle, France). The experiments were conducted in accordance with the guidelines of the European Union (86/609/EU, 2003/65/EU) and Spanish regulations (BOE 252/34367–91, 2005) for the use of laboratory animals in chronic studies and were approved by the Institution Committee for animal care and handling. Selected data collected from these animals have been analyzed for studies of the firing activities of OO Mns (Trigo et al., 1999) and type A and B IPns (Gruart et al., 2000a; Delgado-García and Gruart, 2002, 2005). In the present study, we will concentrate on the comparative analysis (using the cumulative neural integration method, time-intensity dispersion models, multiple parametric evolutions, and raster and 3D representations in the time and frequency domains) of the neuronal firing patterns of the two types of identified cerebellar posterior IPn (types A and B) conforming an agonist–antagonist cerebellar nuclear system for the dynamic control of learned eyelid responses.

SURGICAL PROCEDURES

Animals were anesthetized with sodium pentobarbital (35 mg/kg, i.p.) following a protective injection of atropine sulfate (0.5 mg/kg, i.m.) to prevent unwanted vagal responses. Animals were implanted with a search coil (five turns, 3 mm in diameter) in the center of the left upper eyelid, at ≈2 mm from the lid margin. The coil was made from Teflon-coated multi-stranded stainless steel wire (50 μm external diameter). Coils weighed ≈1.5% of the cat's upper lid weight and did not impair eyelid responses. Animals were also implanted in the ipsilateral OO muscle with bipolar hook electrodes aimed for electromyographic (EMG) recordings. These electrodes were made from the same wire as the coils, and bared 1 mm at their tips.

Four subjects were prepared for the chronic recording of antidromically identified left posterior IPns: a bipolar stimulating electrode, made from 200 μm enamel-coated silver wire, was implanted in the magnocellular division of the right (contralateral) red nucleus following stereotaxic coordinates (Berman, 1968). For comparative purposes, the other four experimental subjects were prepared for the chronic recording of antidromically identified facial Mns projecting to the OO muscle: two stainless steel hook electrodes were implanted on the zygomatic subdivision of the left facial nerve, 1–2 mm posterior to the external canthus (see **Figure 1A**). In each experimental subject, a recording window (5 × 5 mm) was opened in the occipital bone to allow access to the cerebellar posterior interpositus nucleus or facial nucleus. The dura mater was removed, and an acrylic chamber was constructed around the window. The cerebellar surface was protected with a piece of silicone sheet and sterile gauze, and covered with a plastic cap. Finally, animals were provided with a head-holding system for stability and proper references of eyelid coil and recording systems. All the implanted electrodes were soldered to a socket fixed to the holding system. A detailed description of this chronic preparation can be found elsewhere (Trigo et al., 1999; Gruart et al., 2000a; Sánchez-Campusano et al., 2007).

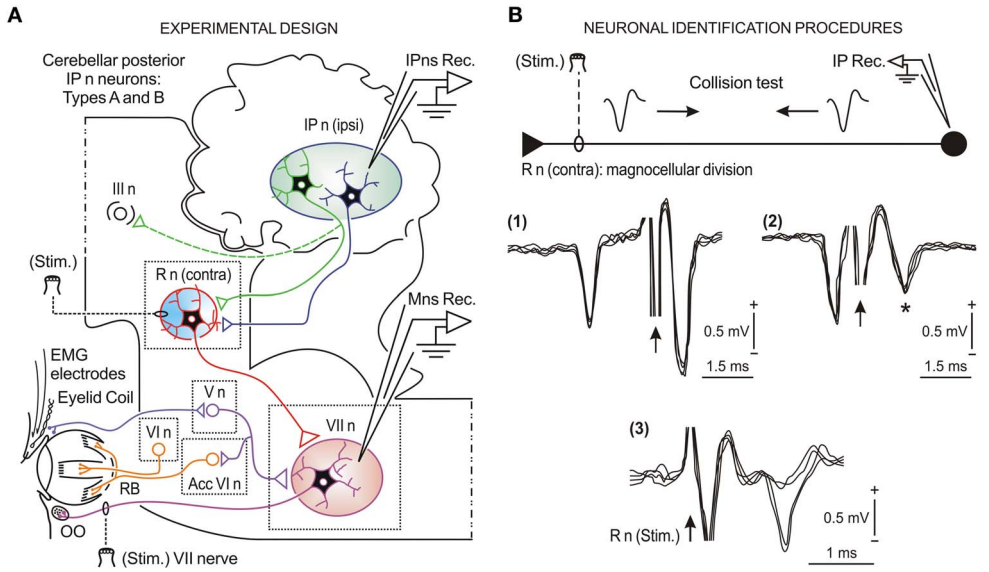

FIGURE 1 | Schematic representation of the experimental design and neuronal identification procedures. (A) Diagram illustrating the stimulating (Stim.) and recording (Rec.) sites, as well as the eyelid coil and EMG electrodes implanted in the upper eyelid. The eyelid kinematics were estimated from the direct recording of the eyelid position by the magnetic field search-coil technique. Kinetic neuronal commands were obtained from the firing activities of antidromically identified neurons (types A and B) located in the ipsilateral cerebellar posterior interpositus nucleus (IP n) and from orbicularis oculi (OO) motoneurons (Mns) located in the facial nucleus (VII n). Abbreviations: R n, red nucleus; III n, oculomotor complex; V n, trigeminal nucleus; VI n, abducens nucleus; Acc VI n, accessory abducens nucleus; RB, retractor bulbi muscle. **(B)** Collision test [**(1)** and **(2)**] of type A and antidromic identification **(3)** of type B interpositus neurons (IPns) activated from the contralateral R n stimulating electrode. The arrows indicate the stimulus artifact. The asterisk in **(2)** indicates that with a stimulus latency of < 0.5 ms the amplitude of the field potential decreased due to the collision of the antidromic spike. Similar procedures were applied for the identification of OO Mns activated from the VII nerve stimulating electrode.

RECORDING AND STIMULATION TECHNIQUES

Eyelid kinematics was recorded with the magnetic field search-coil technique (Gruart et al., 1995). The gain of the recording system was set at 1 V = 10°. The EMG activity of the OO muscle was recorded with differential amplifiers at a bandwidth of 0.1–10 kHz. Neural commands (action potentials and neural firing rates) were recorded in facial and interpositus nuclei with the help of glass micropipettes filled with 2 M NaCl (3–6 MΩ of resistance) using a NEX-1 preamplifier (Biomedical Engineering Co., Thornwood, NY, USA). Neuronal identification techniques, such as antidromic activation (for type B IPns) and collision test (for type A IPns), are illustrated in **Figure 1B** [see sub-panels **(1)**, **(2)**, and **(3)**]. For the antidromic activation of recorded neurons, we used single or double (interval of 1–2 ms) cathodal square pulses (50 μs in duration) with current intensities <300 μA. Identification procedures have been described in detail for facial Mns (Trigo et al., 1999) and posterior IPns (Gruart et al., 2000a; Jiménez-Díaz et al., 2004; Sánchez-Campusano et al., 2007). Only antidromically identified posterior IPns and OO Mns have been included and analyzed in this study.

CLASSICAL EYEBLINK CONDITIONING

The generation of eyelid conditioned responses (CRs) is a slow process requiring a large number of paired conditioned stimulus (CS)/unconditioned stimulus (US) presentations, as we have already described for mice (Domínguez-del-Toro et al., 2004; Gruart et al., 2006; Porras-García et al., 2010), rats (Valenzuela-Harrington et al., 2007; Fernández-Lamo et al., 2009), rabbits (Gruart et al., 2000b; Leal-Campanario et al., 2007), and cats (Gruart et al., 1995, 2000a; Trigo et al., 1999; Sánchez-Campusano et al., 2007). In this study, classical eyeblink conditioning was achieved by the use of a delay conditioning paradigm (see the general structure of the conditioning protocol in **Figure 2A**). A tone (370 ms duration, 600 Hz frequency, and 90 dB intensity) was used as CS. The tone was followed 270 ms from its onset by an air puff (100 ms duration and 3 kg/cm^2 pressure) directed at the left cornea as US. Thus, the CS and the US terminated simultaneously. Tones were applied from a loudspeaker located 80 cm below the animal's head. Air puffs were applied through the opening of a plastic pipette (3 mm in diameter) located 1 cm away from the left cornea.

Each animal followed a sequence of two habituation, 10 conditioning, and three extinction sessions. A conditioning session consisted of 12 blocks separated by a variable (5 ± 1 min) interval. Each block comprised 10 trials separated by intervals of 30 ± 10 s. Within each block, the CS was presented alone during the first trial—i.e., it was not followed by the US. A complete conditioning session lasted for ≈2 h. The CS was presented alone during habituation and extinction sessions for the same number of blocks per session and trials per block and with similar random inter-block and inter-trial distributions (see **Figure 2A**).

HISTOLOGY

At the end of the recording sessions, animals were deeply re-anesthetized (50 mg/kg sodium pentobarbital, i.p.). Electrolytic marks were placed in selected recording sites with a tungsten

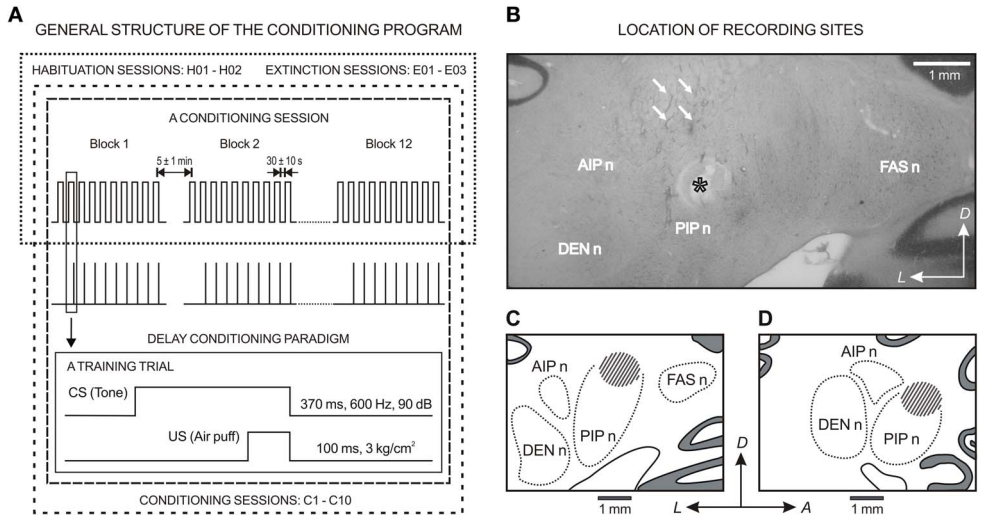

FIGURE 2 | General structure of the conditioning program and location of the recording sites. (A) For classical conditioning of eyelid responses, the experimental animals underwent a sequence of habituation, conditioning, and extinction sessions. In all cases, the session consisted of 12 blocks separated by a varying (5 ± 1 min) interval. Each block consisted of 10 trials separated by intervals of 30 ± 10 s. The conditioned stimulus (CS) was presented alone during habituation and extinction sessions. Classical conditioning responses (CRs) were evoked with the help of a delay paradigm. For this, a tone (370 ms, 600 Hz, 90 dB) was used as CS. The tone was followed 270 ms from its onset by an air puff (100 ms, 3 kg/cm^2) directed at the left cornea as an unconditioned stimulus (US). **(B)** A photomicrograph of a coronal section through deep cerebellar nuclei illustrating several recording tracts (arrows) and an electrolytic mark (asterisk) below recording sites. **(C)** and **(D)** are the coronal and sagittal reconstruction of recording sites (dashed area), respectively. **(B)** and **(C)** correspond to the coronal plane P10, and **(D)** corresponds to the sagittal plane L5.6 according to the atlas of Berman (1968). Calibration bar: 1 mm. Abbreviations: DEN n, dentate nucleus; FAS n, fastigial nucleus; AIP n and PIP n, anterior and posterior interpositus nucleus; *A*, anterior; *D*, dorsal; *L*, lateral.

electrode (1 mA for 30 s). Animals were perfused transcardially with saline and phosphate-buffered formalin. The brain was removed, the anastomosed side was marked and the brainstem and the overlying cerebellum were cut in 50 mm coronal serial sections with the help of a vibratome (WT1000, Leica Microsystems GmbH, Wetzlar, Germany). Serial sections (50 μm) including the cerebellum and the brainstem were mounted on glass slides and stained with toluidine blue or cresyl violet for confirmation of the recording sites (see a photomicrograph of a coronal section through deep cerebellar nuclei and the coronal and sagittal reconstruction of recording sites, **Figure 2B**). Site locations have been described in detail for posterior IPns (Gruart and Delgado-García, 1994; Gruart et al., 2000a) and facial Mns (Trigo et al., 1999).

DATA COLLECTION AND ANALYSIS

Eyelid position, EMG activity of the OO muscle, neuronal activity recorded in facial and cerebellar interpositus nuclei, and rectangular pulses corresponding to CS and US presentations, were stored digitally on a computer, using an analog-digital converter (CED 1401 Plus; Ceta Electronic Design, Cambridge, UK). Commercial computer programs (Spike 2 and SIGAVG; Ceta Electronic Design) were employed for acquisition and on-line conventional analyses. The detailed procedures (including spike detection and sorting, multi-parametric cluster technique, timing correlate, circular time-intensity dispersion method, and frequency domain analysis using the fast Fourier transform), as well as quantification and representation programs were developed by two of us (Raudel Sánchez Campusano and Rodrigo Fernández-Mas) with the help of MATLAB routines (The MathWorks, Natick, MA, USA) and a representation script written in Java language. Only data from successful experiments (i.e., those that allowed a complete study with an appropriate functioning of both recording and stimulating systems) were computed and analyzed (see **Figure 3A**).

The discharge patterns of IPns and OO Mns were analyzed (**Figure 3B**). The algorithm also took into account the identification of the activity's standard waveform and the classification of probability patterns of spikes in time and frequency domains, (Jarvis and Mitra, 2001; Brown et al., 2004; Sánchez-Campusano et al., 2007), and in the phase space (Aksenova et al., 2003). Since raw neuronal recordings usually contain overlapping spikes, we used the following computational procedure: with the application of a spike-sorting method, overlapping spikes within an interval of 1 ms were regarded as a single spike (according to the absolute refractory period) and overlapping spikes within an interval of 1–3 ms were regarded as spikes of different classes due to the interspike interval (i.e., the relative refractory period of the neuron) criterion in spike detection. The cluster tools enabled us to determine the numbers of cells, classes, and spikes and their centers by measuring the distances between their trajectories in the phase space (Porras-García et al., 2010). Spike phase-space reconstruction was implemented using the time-delay technique (Chan et al., 2008), and the reconstructed spike waveform (an ideal and undisturbed spike that can be used as a template for the sorting method) preserves essential characteristics and the major

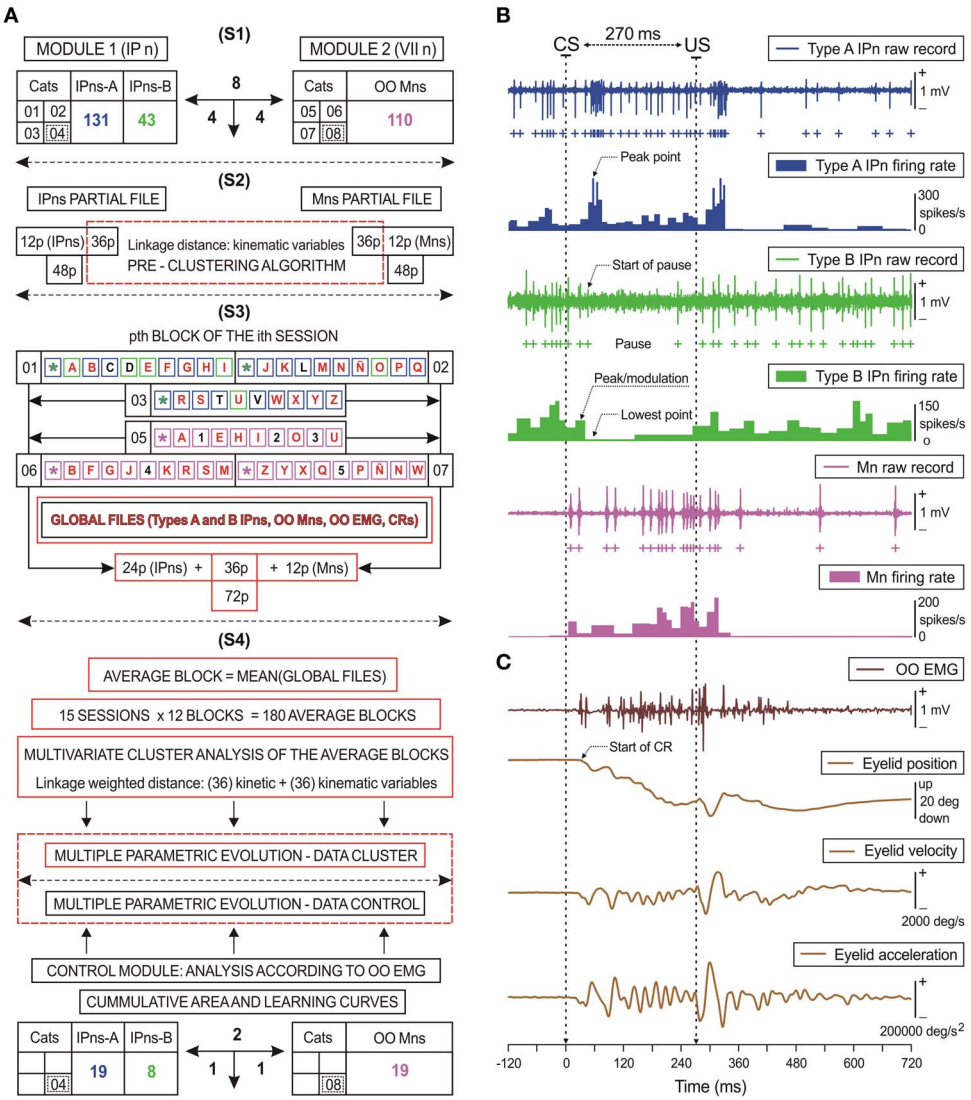

FIGURE 3 | Diagrammatic representation of the data distribution: kinetic neural commands and eyelid kinematics. (A) The first data-processing stage **(S1)** includes all the experimental cats ($n = 8$). Four of the animals were used for recordings at the facial nucleus (VII n), while the other four were used for recordings at the cerebellar posterior interpositus nucleus (IP n). The total number of recorded orbicularis oculi (OO) motoneurons (Mns, $n = 110$), and of type A ($n = 131$) and type B ($n = 43$) interpositus neurons (IPns) are also indicated. In the following stage **(S2)**, a pre-clustering algorithm was applied for 36 eyelid kinematic parameters. In the third stage **(S3)**, an example of the possible combinations (corresponding to the *p*th block of the *i*th session) in order to form adequate global files. Blue, green, and magenta squares represent the trials of the training blocks for the cats 01–03 (types A and B IPns activities) and the cats 05–07 (OO Mns recording). Since for the first trial of each block the conditioned stimulus (CS) is presented alone (.), its possible combinations were not considered. Squares including the same letter (A–Z) represent a combination between an IPns partial file and an OO Mns partial file in order to form a global file per trial. In the illustrated example appeared a total of 27 global files (illustrated by red letters). Black letters and numbers indicate trials in which kinematic properties were significantly different from those in the rest of the trials of the corresponding block and session. Global files were averaged to form the averaged blocks to be further processed with the multivariate cluster analysis including the 72 selected parameters (i.e., 36 kinetic and 36 kinematic variables). In **(S4)** is indicated the correspondence between the multiple parametric evolution for $n = 6$ animals (cats 01–03 and 05–07) and for the control data (cats 04 and 08) using the quantitative analysis of OO EMG activity, the cumulative integration functions, and the learning curves. **(B,C)** A set of recordings collected in the 10th conditioning session from two representative animals. Here are represented the kinetics [neural commands, in **(B)**] and the performance [kinematics, in **(C)**] of eyelid response. **(B)** The action potentials (IPn spikes) marked with blue (type A) and green (type B) plus signs correspond to the direct representation of the neuronal activity in the IP n (IPn raw recordings) and its respective instantaneous frequency (IPn firing rate). Mns spikes recorded from an OO Mn are indicated with magenta plus signs. The direct representation of the neuronal activity in the facial nucleus (Mn raw recordings) and its corresponding instantaneous frequency (Mn firing rate) are also shown. **(C)** These traces illustrate the EMG activity of the OO muscle (OO EMG), the direct recording of the eyelid position by the magnetic field search-coil technique, and the estimated eyelid velocity and acceleration profiles. For each of the physiological signals represented, the magnitude and the respective unit of measurement are indicated. The dotted arrows indicate the peak firing rate of type A IPn (peak point); the start of the pause, the peak firing rate in the modulation range of the pause (peak/modulation), and the lowest firing rate (lowest point) of type B IPn; and finally, the start of the CR.

phase-space trajectory of the original spike. Finally, the instantaneous firing rate was calculated as the inverse of the interspike intervals (see **Figure 3B**).

Maximum eyelid displacements during CRs were determined in the CS–US interval, and the function corresponding to the collected data (frequency sample at 1000 Hz) in the CS–US interval was fitted by a simple regression method. This method enabled fixing the trend for the points near the zero level of eyelid position and establishing a standardized algorithm for all the responses across all the blocks of trials. In this way, the typical randomness in the determination of CR onset was avoided. The onset of a CR (see the dotted line in **Figure 3C**) was determined as the latency from CS presentation to the interception of the regression function with the maximum amplitude level (Sánchez-Campusano et al., 2011b). This method was applied across the successive conditioning sessions, always showing the appropriate precision and robustness. The percentage of CRs was calculated as the ratio (or fraction) between the number of trails that elicited a CR and the total number of CS presentations during a conditioning session (Porras-García et al., 2010; Sánchez-Campusano et al., 2011a). Velocity and acceleration profiles (**Figure 3C**) were computed digitally as the first and second derivatives of eyelid position records after low-pass filtering of the data (−3 dB cut-off at 50 Hz and zero gain at ≈100 Hz) (Domingo et al., 1997; Sánchez-Campusano et al., 2007).

Computed results were processed for statistical analysis using the Statistics MATLAB Toolbox. As statistical inference procedures, both ANOVA and MANOVA (estimate of variance both within-groups and between-groups on the basis of one dependent measure, and estimate of variance in multiple dependent parameters across groups, respectively) were used to assess the statistical significance of differences between groups. The corresponding statistical significance test was performed with sessions as repeated measures, coupled with contrast analysis when appropriate (Hair et al., 1998; Grafen and Hails, 2002). Here we reported the $F_{[(m-1),(m-1)\times(n-1),(l-m)]}$ statistics and the resulting probability $P < 0.05$, in which, 0.05 was the maximum predetermined significance level for all of the tests. The orders m (number of groups), n (number of animals), and l (number of multivariate observations) and the corresponding degrees of freedom were reported accompanying the F statistics values. According to the F-distribution, if the probability P is less than the predetermined significance level (0.05), then we reject the null hypothesis (no difference between population means). In contrast, if $P > 0.05$ we do not reject the null hypothesis at the significance level of 0.05.

Wilk's lambda criterion and its transformation to the χ^2-distribution used in MATLAB were applied to infer the existence of statistically significant differences between samples from MANOVA results: (1) cluster analysis for cells-classes-spikes classification during the spike-sorting problem in the phase space (Porras-García et al., 2010) and (2) hierarchical cluster-free reconstruction during the kinetic-kinematic characterization of the learning process (Sánchez-Campusano et al., 2007, 2011b). For the circular statistics, we used both the Rayleigh and the Watson hypothesis tests for the *von Mises distribution* (Fisher, 1993; Jammalamadaka and SenGupta, 2001; Berens, 2009). Readers may refer to Sánchez-Campusano et al. (2011b) for a detailed and practical description of this circular statistics technique to analyze timing and time-dispersion patterns during motor learning.

RESULTS

We recorded and analyzed a total of 174 antidromically identified cerebellar posterior IPns. From their discharge properties, we classified 131 of these 174 neurons as type A IPns and the remaining 43 neurons as type B IPns (**Figure 3A**). Type A IPns increase their firing frequency in the CS–US interval across successive conditioning sessions (Gruart et al., 2000a; Sánchez-Campusano et al., 2007), whilst type B IPns pause or even stop firing in the CS–US interval during the same conditioning paradigm (Gruart and Delgado-García, 1994; Gruart et al., 2000a). In addition, we recorded and analyzed 110 antidromically identified OO Mns (**Figure 3A**). Characteristically, OO Mns encode eyelid position during CRs (Trigo et al., 1999; Sánchez-Campusano et al., 2009). The two pools of neurons (IPns and OO Mns) were recorded in separate experiments in behaving cats during classical eyelid conditioning using a delay paradigm. However, and in accord with a previous study (Sánchez-Campusano et al., 2007), the kinetic–kinematic characterization of the recordings (involving the OO Mns and IPns activities and the eyelid responses) and the multivariate cluster analysis of the collected data enabled us to determine the distribution of homogeneities across the training blocks and sessions and the intrinsic coherence of recorded parameters (kinetic and kinematic variables) regarding the actual learning process.

The present study focuses on the comparison of the firing properties of OO Mns and type A IPns to those of type B IPns (**Figure 3A**). We analyzed the experimental data collected across the successive training sessions using cumulative the neural integration method, time-intensity dispersion models, multiple parametric evolutions, and raster and 3D representations in the time and frequency domains. As a result, the optimized global files were clustered automatically (see an example of a global file in **Figures 3B,C**) taking into account physiological criteria (see a complete list of kinetic and kinematic parameters in Sánchez-Campusano et al., 2007).

FIRING PROPERTIES OF THE POSTERIOR INTERPOSITUS NEURONS DURING CLASSICAL CONDITIONING OF EYELID RESPONSES

In those animals ($n = 4$) prepared for recording the firing activity of identified IPns, deep cerebellar nuclei were systematically explored in order to find unitary activity related with eyelid responses. The unitary activity was recorded during the random presentation of air puffs (10–100 ms, 1–3 kg/cm^2) aimed at the ipsilateral cornea (**Figure 1A**). This procedure took ≤ 5 recording sessions. As confirmed later by the histological study, the dorsomedial part of the posterior interpositus nucleus was found to contain a high density of neurons related to reflexively evoked eyelid responses. Recorded neurons were identified by their antidromic activation (latency of 0.5–1.22 ms) from the contralateral red nucleus (**Figures 1A,B**). Although other deep cerebellar nuclei (mainly anterior interpositus and dentate nuclei) also contain neurons related to eyelid movements, we consider here only those located in the indicated area of

the posterior interpositus nucleus (**Figures 2B–D**). Once the recording area was located, animals were classically conditioned using a delay paradigm. Posterior IPns were recorded in the four animals across two habituation, 10 conditioning, and three extinction sessions. The mean number of neurons recorded per session was 2.9 (i.e., a mean of 41.37 min of recording per neuron).

Type A IPns ($n = 131$) fired during active contraction of the OO muscle, and therefore, during the downward movements (either conditioned or unconditioned eyelid responses) of the upper eyelid (see blue profiles in **Figure 3B**). Tones presented during the two habituation sessions evoked relatively few reflex eyelid responses, which appeared mainly during the first blocks of the first habituation session. During habituation sessions, IPn firing lagged the beginning of evoked eyelid responses by 12–30 ms, but the mean value of this lag decreased progressively across conditioning (Gruart and Delgado-García, 1994; Gruart et al., 2000a; Sánchez-Campusano et al., 2007, 2009). The mean number of spikes generated by type A IPns in the CS–US interval did not change significantly and their mean peak firing rate increased across conditioning and decreased during extinction sessions (for more details see below, the multiple parametric evolutions). According to previous reports, type A IPns exert a reinforcing-modulating action on the OO Mns (via the red nucleus) during the closure of the eyelid (Sánchez-Campusano et al., 2007, 2009, 2011a,b).

Type B IPns ($n = 43$) were also antidromically activated (**Figure 1B**) from the red nucleus (0.85–1.25 ms), but presented a noticeable inhibition in their firing (see green profiles in **Figure 3B**) in coincidence with the downward displacement of the upper eyelid during reflexively evoked blinks (Gruart and Delgado-García, 1994; Gruart et al., 2000a). The firing rate of the type B IPns began to decrease (start of pause), a few milliseconds (<10 ms) before the onset of the CR. However, their firing rate reached the minimum value of amplitude (the lowest point) at 8–25 ms after the onset of the CR (see the dotted arrows in **Figures 3B,C**). In general, the duration of the pause (including the modulation range) in the activity of type B IPns was from −10 ms to +200 ms with respect to the beginning of the CR. Thus, the main functional difference between type A and type B IPns was that type A neurons increased their firing in the CS–US interval across successive conditioning sessions, whilst type B IPns paused or even stopped firing in the CS–US interval during the same conditioning paradigm.

INTEGRATED NEURAL ACTIVITIES OF ORBICULARIS OCULI MOTONEURONS AND TYPES A AND B CEREBELLAR POSTERIOR INTERPOSITUS NEURONS

In the other group of animals ($n = 4$), the facial nucleus was systematically explored in the search for OO Mns antidromically activated from the electrode implanted in the zygomatic branch of the facial nerve (**Figure 1A**). As already described (Shaw and Baker, 1983, 1985; Gruart et al., 2003) and confirmed histologically, OO Mns are concentrated in the dorsal subdivision of the facial nucleus. These OO Mns ($n = 110$) were antidromically activated, with a mean latency of 2.19 ± 0.38 ms (mean ± SEM), and their firing started ≈10 ms before air-puff-evoked eyelid reflex responses. Once the recording area was located, animals were classically conditioned as indicated above (**Figure 2A**). OO Mns fired tonically (magenta profiles in **Figure 3B**) during the performance of the eyelid CRs (see eyelid position, velocity, and acceleration profiles in **Figure 3C**). Thus, and as already described (Trigo et al., 1999), their discharge was related to eyelid position (with a gain of 4.5–11.3 spikes/deg; $r \geq 0.92$; $P \leq 0.01$) during CRs. Interestingly, the total number of spikes generated by OO Mns during the CS–US interval increased across conditioning (see below, the multiple parametric evolutions).

To determine the correlation degree between the discharge rate of facial Mns and that of IPns, we compared the cumulative numerical integration functions obtained as the sum of the trapezoidal integrals of the firing frequencies across the CS–US interval. **Figure 4A** illustrates the integrated neural activities of three representative interpositus nucleus neurons (IPn1–IPn3) and a representative OO Mn during the 10th conditioning session. It should be noted that all the integrated neural activities showed some slight and relative variations around the local and global maximum instantaneous frequency values. We defined three equivalence times (L1–L3) at the points where IPn2 and OO Mn traces crossed.

The presence of an oscillatory behavior in the firing of IPns and OO Mns was evidenced by high-pass filtering the traces illustrated in **Figure 4A**. The relative variation functions obtained after the high-pass filtering process presented oscillatory and phase-inversion properties (**Figure 4B**). Note that on the oscillating curves shown in **Figure 4B**, components 1–4 of the type A IPn are out of phase with components 5–8 of the OO Mn. These results allow proposing a modulatory role for type A IPns in the final common pathway for the eyelid system (i.e., the motoneuronal pool) which, by progressively inverting phase information, modulate or reinforce eyelid motor responses inversely—not opposed—to the contribution of OO Mns (Sánchez-Campusano et al., 2007, 2009). Furthermore, the maximum amplitude of the OO Mns relative variation function was significant [One-Way ANOVA F-tests, $F_{(9,27,98)} = 170.26$, $P < 0.01$] both in the CS–US interval and after the US presentation. Finally, the oscillation amplitude of the type A IPn relative variation function increased progressively across the learning process, reaching significant values [One-Way ANOVA F-tests, $F_{(9,27,98)} = 59.51$, $P < 0.01$] during the 10th conditioning session (**Figure 4B**).

In **Figure 4C**, we illustrate the integrated neural activity of type B IPns (IPn4, green trace) in comparison with that of type A IPns (IPn2, blue trace). Here, we defined two equivalence times (L4 and L5) at the points where IPn2 (type A) and IPn4 (type B) traces crossed. Note that the times of equivalence between types A and B IPns are smaller than those between type A IPns and OO Mns (i.e., L4 < L1 and L5 < L2). Thus, the range of initial modulation of the pause (i.e., the decrease of firing rate of type B cells before they stop firing altogether) was from L4 to L5 ms. The main outcomes of this method were the recognition of the cumulative profiles of the neural activities (types A and B IPns) with a definite dissimilarity in the cumulative areas of their firing frequencies, and the identification of a temporal similarity in the range of occurrence of both the peak (in type A IPns) and the lowest (in type B IPns) firing frequencies

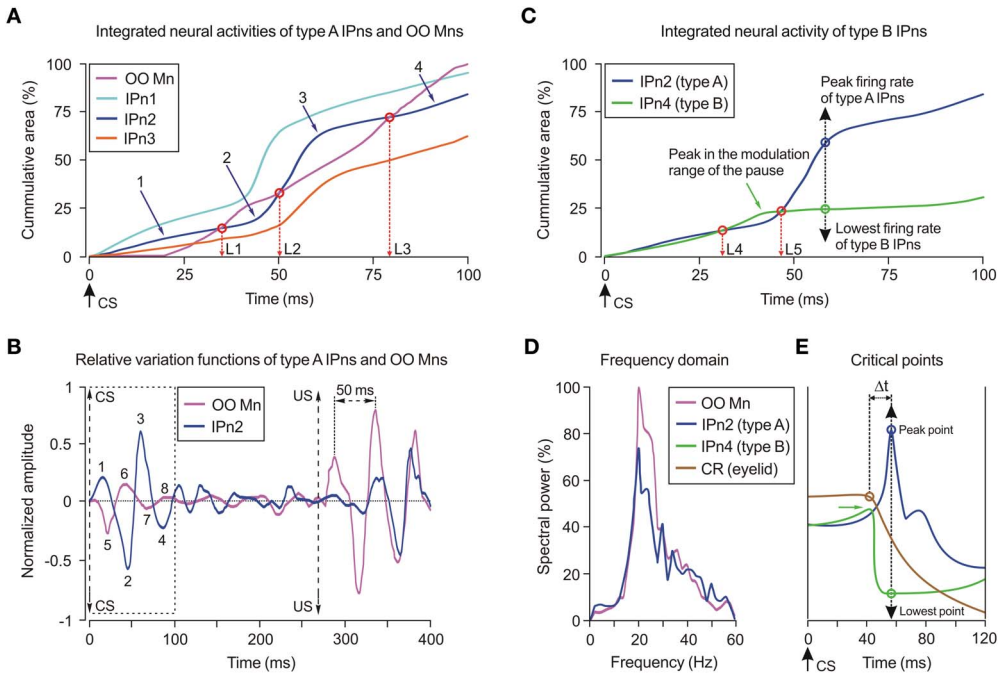

FIGURE 4 | Cumulative neural integration functions of the types A and B cerebellar posterior interpositus neurons. (A) Three of the illustrated traces correspond to the averaged integrated neural activity of identified type A cerebellar interpositus neurons (IPn1–IPn3, see color codes in the inset), while the fourth trace represents the integrated neural activity of a selected orbicularis oculi motoneuron (OO Mn, see code in the inset). Data were collected from the 10th conditioning session. Here, the CS presentation (time 0 ms) and the equivalence times (L1, L2, and L3) of integrated neural activities (IPn2 and OO Mn) are indicated. **(B)** Oscillatory and phase-inversion properties of type A IPn (e.g., IPn2). Oscillatory curves (relative variation functions) resulting from high-pass filtering (−3 dB cutoff at 5 Hz and zero gain at ≈15 Hz) of integrated neural firing activities illustrated in **(A)**. In **(B)**, OO Mn and IPn2 relative variation functions present a phase difference during their oscillations in the CS–US interval. Here, the components 1, 2, 3, and 4 of the type A IPn2 are totally out of phase with the components 5, 6, 7, and 8 of the OO Mn, respectively. **(C)** Integrated neural activity of a type B IPn (e.g., IPn4) in comparison with the type A IPn2. Note that the green trace (IPn4) increases, though only slightly, for time values > 40 ms [temporal range of the pause (including the modulation range) in the firing activities of the type B IPn]. The dashed black double arrow indicates the peak (type A, blue circle)/lowest (type B, green circle) firing frequency. **(D)** Power spectra for the oscillating curves shown in **(B)**. Note that the two illustrated spectra present a significant predominance of spectral components at ≈20 Hz, and significant differences [One-Way ANOVA *F*-tests, $F_{(1,3,238)} = 20.11$, $P < 0.01$] between their spectral powers. **(E)** The critical points (peak and lowest firing frequencies of types A and B IPns, respectively) of the numerical distribution functions of the envelope of the firing rates (type A IPns, blue curve; type B IPns, green curve) in the 0–120 ms interval. The distribution function in brown corresponds to the eyelid position profile and the brown circle indicates the latency to CR onset. The delay Δt ≈16 ms between the critical points and the time to CR onset during the 10th conditioning sessions is also indicated.

(see the dashed black double arrow in **Figure 4C**) in the CS–US interval.

The illustrated power spectra (**Figure 4D**) presented a significant predominance of spectral components at ≈20 Hz [20.13 ± 0.04 Hz, (mean ± SEM)], and significant differences in their spectral power [One-Way ANOVA *F*-tests, $F_{(1,3,238)} = 20.11$, $P < 0.01$] at the asymptotic level of acquisition of this associative learning test (session C10). We also found significant differences in the power spectra of Mn [One-Way ANOVA *F*-tests, $F_{(9,27,98)} = 225.48$, $P < 0.01$] and type A IPn [One-Way ANOVA *F*-tests, $F_{(9,27,98)} = 216.28$, $P < 0.01$] physiological time series across conditioning sessions. The integrated neural activity of type B IPns did not show oscillatory properties because the pause in their firing activity in the CS–US interval annulled the relative variation of amplitude.

The time-dependent distribution functions of the integrated neural activities (types A and B IPns, blue and green curves, respectively) and the integrated eyelid position (brown trace) are represented in **Figure 4E**. Here, we show the critical points (maximum and minimum points of the distributions) in correspondence with the peak and lowest firing frequencies of the type A and B IPns (see the dashed black double arrow in **Figures 4C,E**). Note that the latency to CR onset [for the brown circle, 41.75 ± 0.56 ms (mean ± SEM)] was smaller than the latencies of the critical points (57.61 ± 0.13 ms) during the 10th conditioning session—i.e., the time to peak (type A IPns)/lowest (type B IPns) firing rates with respect to CS onset always lagged (Δt ≈16 ms) the beginning of the CR.

DYNAMIC NEURAL PATTERNS OF TYPES A AND B INTERPOSITUS NEURONS AND MULTIPLE PARAMETRIC EVOLUTIONS ACROSS CONDITIONING

It has been proposed that learning is a precise functional state of the brain, and that we should take a dynamic approach to the study of neural activity during learning in alert behaving animals to determine whether this is so (Delgado-García and Gruart,

2002; Sánchez-Campusano et al., 2011a). Here, we show a comparative analysis of the dynamic neural patterns of the two types of identified cerebellar posterior IPn using raster and 3D representations of the firing activity in the time domain (**Figures 5A,B** and **6A,D**), multiple parametric evolutions (**Figures 5C–E**), and raster and 3D representations of the eyelid kinematics in the time (**Figures 6B,E**) and frequency (**Figures 6C,F**) domains.

In previous studies (Gruart et al., 2000a; Sánchez-Campusano et al., 2007), we analyzed the time-course of the firing activity of type A IPns across conditioning sessions. In the present study, we have analyzed the dynamic evolution of the firing activity of type A IPns using the intra-trial (e.g., **Figures 3B,C**) and inter-trials (**Figure 5A**) timing schemes. **Figure 5A** (raster representation) and **Figure 5B** (3D-representation) show the profiles (color map and surface plots) of the firing rate (in spikes/s) of type A IPns. The main outcome of the raster representation was the identification of the temporal range of the burst (including the modulation range) in the activity of type A IPns (e.g., the time

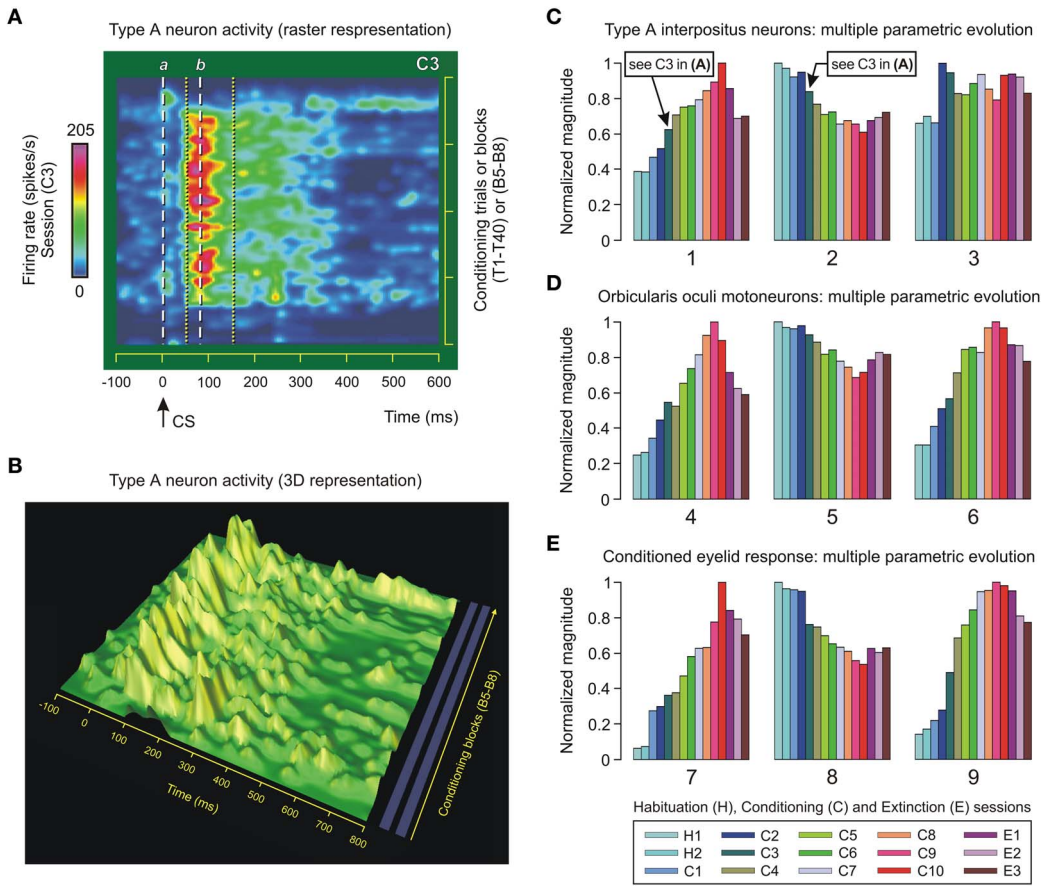

FIGURE 5 | Type A neuron activity in the interpositus nucleus and multiple parametric evolution across conditioning. (A) Color raster display of the firing rate (in spikes/s, see the quantitative color bar to the left of the panel) of a representative type A cerebellar posterior interpositus neuron (IPn) recorded during the third conditioning session (C3) using the delay paradigm. The trials T1–T40 correspond to the conditioning blocks B5–B8 of session C3. Neuronal firing rates of only one in three conditioning trials are represented. The dashed white lines labeled *a* and *b* correspond to conditioned stimulus (CS) presentation (line *a*, 0 ms) and ≈79 ms afterwards—that is, the mean value of the latency (line *b*) to peak firing rate of the type A IPns in the inter-stimulus interval (ISI). The dotted yellow lines indicate the temporal range of the burst (including the modulation range) in the firing activities of type A IPns. **(B)** 3D representation of the dynamic evolution of the firing frequency profiles of type A IPn for the same sequence of conditioning trials and blocks as in the panel **(A)**. Here, the time (in ms), the block of conditioning trials, and the firing rate of type A IPn (in spikes/s) are represented on the *x*, *y*, and *z*-axes, respectively. **(C–E)** A representation of multiple parametric evolutions of physiological parameters collected across conditioning. The color code indicates the corresponding session, and each set of colored bars corresponds to the evolution of a given parameter (numbered from 1 to 9): parameters 1 and 4 (mean peak firing rate, in spikes/s), parameters 2 and 5 (latency of the mean value of the peak firing frequency with respect to CS presentation, in ms), parameters 3 and 6 (total number of spikes in the ISI), parameter 7 [eyelid position amplitude at unconditioned stimulus (US) presentation compared with the amplitude at the start of the conditioned response (CR), in degrees], parameter 8 (latency between the CS and the onset of the CR, in ms), and parameter 9 (the typical learning curve, in %CR). The timing (2 and 5) and kinetic (1, 3, 4, and 6) parameters represented in **(C)** and **(D)** were calculated from both IPn and OO Mn recordings, respectively. The arrows in **(C)** indicate the mean value of the peak firing frequency (0.62—that is, ≈201 spikes/s) and its time of occurrence (0.84—that is, ≈78 ms) during the conditioning session C3. The above values are in correspondence with those represented in **(A)** (see the color bar for the peak firing rate and the line *b*). The parameters 7, 8, and 9 in **(E)** characterize the time-course and kinematics of the learned eyelid response. For this representation, each parameter has been normalized in accordance with its maximum value across conditioning.

interval between the dotted yellow lines during the third session, **Figure 5A**). Here, the latency to peak firing rate of type A IPns was 79.42 ± 0.37 ms (mean $\pm$ SEM) (see the dashed white line *b* in **Figure 5A**). This latency decreased progressively across conditioning sessions with respect to CS presentation (see below, the multiple parametric evolutions).

The organization of data in the matrix provided by multivariate cluster analysis allowed the selection of the most-significant physiological parameters representing the acquisition and extinction processes. Nine representative parameters (timing, neural, and kinematic variables), numbered from 1 to 9, are depicted in **Figures 5C–E**. In **Figure 5C**, we show the maximum instantaneous frequency of type A IPns in the CS–US interval (parameter 1), the latency of their peak firing frequency with respect to CS presentation (parameter 2), and the mean number of spikes generated by type A IPns in the CS–US interval (parameter 3). Note that the parameter 1 [One-Way ANOVA *F*-tests, $F_{(14,70,132)} = 143.86$, $P < 0.01$] increases across the conditioning sessions. The irregular evolution of parameter 3 [One-Way ANOVA *F*-tests, $F_{(14,70,132)} = 1.63$, $P > 0.05$] suggests that the increase in parameter 1 after CS presentation represented a reorganization (rather than a net increase) of the mean spontaneous firing of type A IPns. In fact, the mean values for the relative refractory period of type A IPns [One-Way ANOVA *F*-tests, $F_{(14,70,132)} = 126.44$, $P < 0.01$] and parameter 2 [One-Way ANOVA *F*-tests, $F_{(14,70,132)} = 93.87$, $P < 0.01$] decrease in the CS–US interval.

As illustrated in **Figure 5D**, the mean peak firing rate [parameter 4, One-Way ANOVA *F*-tests, $F_{(14,70,132)} = 207.31$, $P < 0.01$] and total number of spikes generated by OO Mns during the CS–US interval [parameter 6, One-Way ANOVA *F*-tests, $F_{(14,70,132)} = 187.12$, $P < 0.01$] increase across conditioning sessions. This result indicates that the dorsolateral portion of the facial nucleus (the site where OO Mns are located) was involved as the neural element (kinetic neural command) driving the eyelid CRs. An inverted evolution (from long to short periods or latencies) was obtained for the mean values of the relative refractory period of the OO Mns [One-Way ANOVA *F*-tests, $F_{(14,70,132)} = 206.20$, $P < 0.01$] and for the latency (with respect to CS onset) of their maximum instantaneous frequency [parameter 5, One-Way ANOVA *F*-tests, $F_{(14,70,132)} = 53.19$, $P < 0.01$] across conditioning sessions.

The representative kinematic parameters (performance of learned eyelid response) are represented in **Figure 5E**. The peak amplitude of the evoked CR [parameter 7, One-Way ANOVA *F*-tests, $F_{(14,70,132)} = 251.27$, $P < 0.01$] increased steadily across conditioning sessions and decreased progressively during the three extinction sessions. However, the mean values of the latency between CS onset and the start of the CR decreased, with significant statistical differences [parameter 8, One-Way ANOVA *F*-tests, $F_{(14,70,132)} = 123.50$, $P < 0.01$], along the conditioning process. The typical learning curve (i.e., the percentage of CRs across conditioning) is also represented [parameter 9, One-Way ANOVA *F*-tests, $F_{(14,70,132)} = 129.40$, $P < 0.01$].

In summary, in **Figures 5C–E** are illustrated the multiple parametric evolutions. These representations are analogous to the one observed in typical learning curves using the classical conditioning paradigm, i.e., a curve showing that the level of expression of CRs increases across the successive conditioning sessions and decreases during the extinction sessions (e.g., parameters 1, 4, 6, 7, and 9) or vice versa (e.g., parameters 2, 5, and 8). Here, the 10th session is the asymptotic level of acquisition of this associative learning test, and the extinction is like a (new) learning process with opposite effects on the level of expression of the responses.

Finally, the parameters were normalized in accordance with their maximum values across conditioning (**Figures 5C–E**). For example, the maximum values for mean peak of the firing frequency were 322.60 spikes/s (parameter 1, session C10) and 158.27 spikes/s (parameter 4, session C9) for type A IPns and OO Mns, respectively. Note that in the session C3, parameter 1 was 201.11 spikes/s. With regard to the mean latency to the maximum instantaneous frequencies, the maximum values were 93.06 ms (parameter 2, session H1) and 259.14 ms (parameter 5, session H1) for type A IPns and OO Mns, respectively. Here, during the session C10, parameter 2 was 56.81 ms (e.g., the dashed black double arrow in **Figures 4C,E**), and during the session C3 it was 77.90 ms (e.g., the line *b* in **Figure 5A**). Furthermore, the maximum values for mean number of spikes generated during the CS–US interval (parameters 6 and 9) were 15.38 spikes (in session C2) and 9.83 spikes (in session C9) for type A IPns and OO Mns, respectively.

In this study we paid special attention to the dynamic neural patterns of type B IPns. **Figures 6A,D** illustrate the time-course of the firing rate (in spikes/s) across four selected conditioning sessions (e.g., C3–C6) in a representative animal. Note that during the conditioning sessions C3–C6, the duration of the pause (including the modulation range) in the firing activity of type B IPns was ≤ 100 ms (from 50 ms to 150 ms with respect to CS presentation, see dotted yellow lines in **Figure 6A**), but this range of duration increased progressively from the seventh to the tenth conditioning session. It was evident from simple observation of the firing records obtained at the asymptotic level of acquisition (i.e., the 10th conditioning session) of this associative learning test (see **Figure 3B**) that the type B IPns pauses and their firing frequency decreased in a specific temporal range (i.e., from 40 ms to 260 ms with respect to CS presentation). Interestingly, during the conditioning sessions C3–C6, the mean value for the latency to lowest point of the firing rate of type B IPns in the CS–US interval was 80.36 ± 0.11 ms (mean $\pm$ SEM) after the presentation of the CS (see the dashed white lines *a* and *b* in **Figure 6A**). Note that a similar mean value (79.42 ± 0.37 ms) was obtained for the latency to peak firing rate of type A IPns during the third conditioning session (**Figure 5A**).

The color raster display and 3D-plot of the eyelid acceleration profiles during the same conditioning sessions (C3–C6) are represented in **Figures 6B,E**. The observed pattern is characteristic of an oscillatory process describing the quantal organization of reflex and conditioned eyelid responses (Domingo et al., 1997). According to the raster representation, the latency to onset of the CR was 60.45 ± 0.28 ms (mean $\pm$ SEM) (see dashed white line *c* in **Figure 6B**) after the presentation of the CS (dashed white line *a*). Therefore, the latency of the lowest amplitude of the instantaneous frequency of type B IPns (line *b*

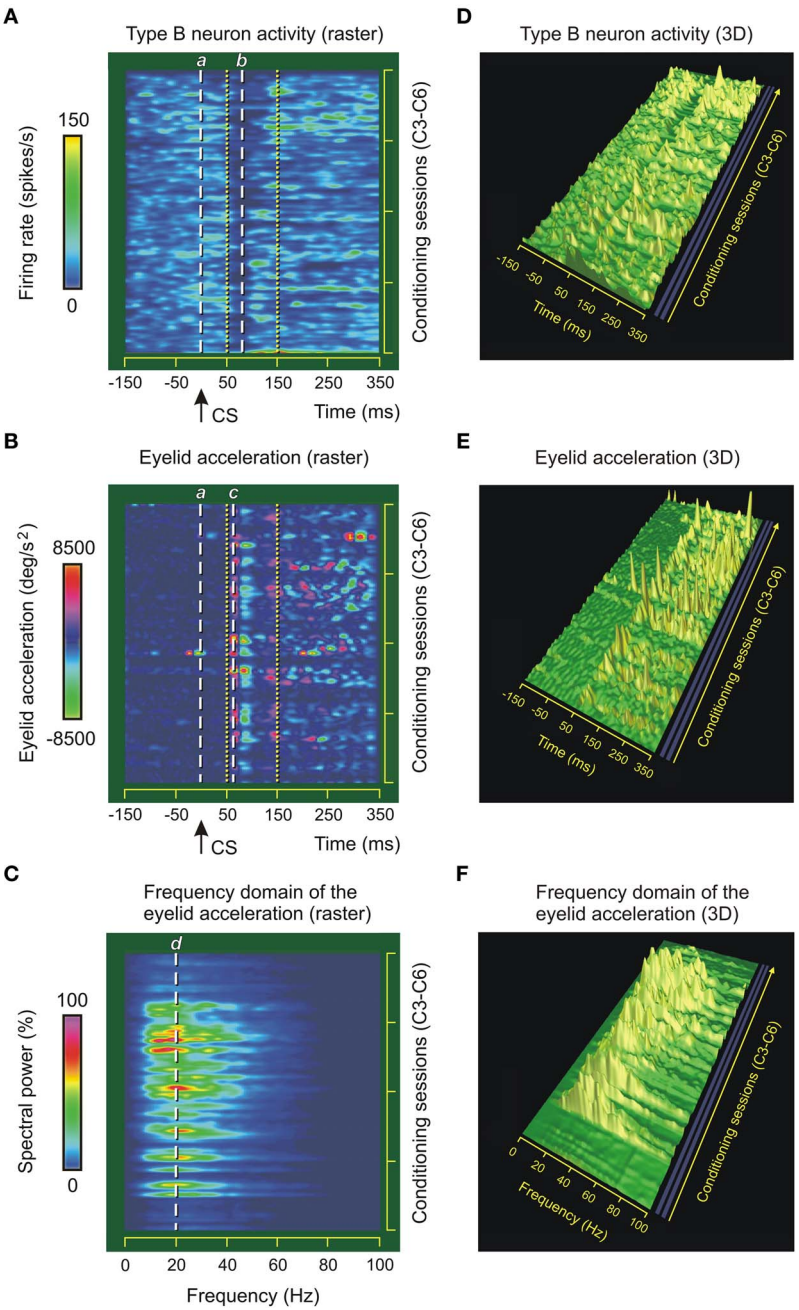

FIGURE 6 | Type B neuron activity in the interpositus nucleus and eyelid kinematics. (A) Color raster display of the firing rates (in spikes/s) of different type B cerebellar posterior interpositus neurons (IPns, $n = 9$) of a representative animal recorded during four conditioning sessions (C3–C6) using the delay paradigm. The dashed white lines labeled *a* and *b* correspond to conditioned stimulus (CS) presentation (0 ms) and ≈80 ms afterwards—i.e., the mean value of the latency (line *b*) to lowest point of the firing rate of the type B IPns in the inter-stimulus interval. **(B)** Color raster display of the eyelid acceleration profiles during the same conditioning sessions (C3–C6). The dashed white line *c* represents the latency between the CS (line *a*, 0 ms) and the onset of the conditioned response (CR) ≈60 ms. The lines *a*, *b* in **(A)** and *c* in **(B)** indicate instantaneous events taking place at the indicated times. Thus, the temporal difference *b*–*c* ≈20 ms allowed us to verify that the latencies of the lowest amplitudes of the instantaneous frequencies (in spikes/s, see line *b*) of type B IPns always lagged the beginning of the CRs. The dotted yellow lines in **(A)** and **(B)** indicates the temporal range of the pause (including the modulation range) in the firing activities of type B IPns. **(C)** Color raster display of the spectral powers (in %) of the eyelid acceleration profiles across conditioning sessions. Note that the frequency domain representation presents a predominance of spectral components at ≈20 Hz—i.e., a predominance of oscillations of eyelid acceleration at ≈50 ms in the time domain. For a more exhaustive interpretation of these raster representations, see the quantitative color bar to the left of each panel. **(D–F)** 3D representation for the dynamic evolutions of the profiles of the firing rates of the type B IPns in **(A)**, the eyelid accelerations in **(B)**, and their spectral powers in **(C)**, respectively. For all the 3D-plots, the same sequence of conditioning trials, blocks, and sessions was used as in **(A–C)** (i.e., profiles of only one in three conditioning trials are represented).

in **Figure 6A**) always lagged the beginning of the CR (line *c* in **Figure 6B**).

Figures 6C,F illustrates the frequency domain analysis of the eyelid acceleration profiles shown in **Figures 6B,E**. The predominance of spectral components observed around 20 Hz [19.51 ± 0.09 Hz, (mean $\pm$ SEM), see **Figure 6C**] was probably due to the 50 ms mean period characteristic of eyelid kinematics in the time domain. In fact, an oscillation at the same dominant frequency ($\approx$20 Hz) has been observed in the relative variation functions of both OO Mns and eyelid position (Sánchez-Campusano et al., 2007, 2011b) and in the EMG activity of the OO muscle (Domingo et al., 1997). It should also be noted that the mean values of the spectral powers (in %, **Figures 6C,F**) increase across the represented conditioning sessions in parallel with the peak amplitudes of the evoked CRs (see parameter 7 in **Figure 5E**).

TIMING AND TIME-INTENSITY DISPERSION PATTERNS OF TYPES A AND B INTERPOSITUS NEURONS DURING MOTOR LEARNING

A set of techniques referred to as circular statistics has been developed for the analysis of directional data (Batschelet, 1981; Fisher, 1993; Jammalamadaka and SenGupta, 2001; Berens, 2009; Sánchez-Campusano et al., 2011b). The unit of measurement for such data is angular (usually in either degrees or radians) and the circular distributions underlying the techniques are characterized by the proper time-angle and intensity-radius correspondences. In this paper, we adapted this approach to analyze the time-intensity dispersion patterns of the collected data in the 0–270 ms interval (the duration of ISI—i.e., the CS–US interval) during the performance of the CR: for example, the angle of 0 degrees is deemed to correspond to a time of 0 ms—that is, the CS onset instant; and the angle of 270 degrees is deemed to correspond to the time of US presentation—that is, 270 ms after CS onset, according to our delay paradigm (**Figure 2A**). Here, we complete three simple steps: (1) timing data were expressed as angles in radians and intensity data were normalized in accordance with their maximum value across conditioning (i.e., circumferences of radios $\leq$1); (2) the corresponding elements of data stored in polar coordinates (angle, radius) were transformed to Cartesian coordinates (x, y); and, (3) the compass plot with components (x, y) as arrows of different lengths emanating from the origin was implemented. In **Figures 7A,B** we show the circular distributions (using the compass plot representation) of our physiological data across conditioning sessions.

In **Figure 7A**, we selected as the timing components of the distributions the time to CR onset (see brown arrows) and the time to peak firing rate of the type A IPns (see blue arrows). The intensity components of the distributions were the percentage of CRs and the peak firing rate of type A IPns, respectively. Here, the timing-intensity associations enabled us to illustrate the simultaneous evolution of the timing and intensity components of the data distributions (from OO Mns and type A IPns firing activities, and eyelid CRs). Note the inverse interrelations between the percentage of CRs and the time to CR onset (brown arrows), and between the peak firing rate of type A IPns and their corresponding time of occurrence (blue arrows) across this associative learning test.

The right-hand circumferences in **Figure 7A** and the circular sectors in **Figure 7C** show the relative dispersion patterns of the time-intensity distributions. For example, in **Figure 7A** the mean values of the time to peak firing rate of type A IPns across the conditioning sessions [blue arrows, $\sigma s = 10.92 \pm 1.01$, (mean $\pm$ SEM)] were less spread out than the mean values of either time to CR onset [brown arrows, $\sigma s = 14.94 \pm 1.23$, (mean $\pm$ SEM)] or time delay in coupling between type A IPn firing frequency and eyelid position response [red arrows, $\sigma s = 45.84 \pm 2.48$, (mean $\pm$ SEM)]. These time-intensity patterns allowed us to verify the previous results,—i.e., the time to peak firing rate of type A IPns always lagged the beginning of the CRs [blue arrows, mean timing $\overline{T}s = 67.64 \pm 3.07$ ms, (mean $\pm$ SEM); brown arrows, mean timing $\overline{T}s = 52.62 \pm 1.89$ ms, (mean $\pm$ SEM)]. Interestingly, the dispersion of the time delay of the correlation (type A IPns vs. eyelid position) showed a significantly [One-Way ANOVA F-tests, $F_{(9,27,98)} = 223.54$, $P < 0.01$] longer transition from larger to smaller values, than did the time to peak firing rate of type A IPns across the sessions. Thus, to the beginning of the learning process the type A IPns encoded (from moderate to weak correlation) eyelid position responses after reaching their maximum firing rate, but at the end of the process (i.e., at the asymptotic level of acquisition of this associative learning test) the IPns encoded (with barely significant correlation) eyelid kinematics before their peak firing rate (but always after the beginning of the CRs). In geometric terms, the centroid of the blue circular sector (corresponding to the time to peak firing rate of the type A IPns in **Figure 7C**) was much further away from the center of the circumference than the centroid of the red circular sector [corresponding to time delay of the correlation (type A IPns vs. eyelid position)] was from the center of the same circumference—that is, the index of circular spread of the blue circular sector [$\sigma = 5.77 \pm 0.56$; $\overline{T} = 69.74 \pm 2.26$ ms, (mean $\pm$ SEM)] was smaller than the time-dispersion index of the red circular sector [$\sigma = 32.71 \pm 1.16$, $\overline{T} = 77.48 \pm 3.05$ ms, (mean $\pm$ SEM)]. This is generally the case—data sets with a greater degree of dispersion have centroids closer to the center of the circumference.

In the same way, in **Figures 7B,D** we illustrate the time-intensity distributions for the type B IPns (see the green and orange arrows). Here, we selected as the intensity components of the distributions the peak firing rate of type B IPns in the modulation range of the pause (parameter 10 in **Figure 7E**) and the lowest firing frequency of type B IPns during the pause. The timing components of the distributions were the time to peak firing rate of type B IPns in the modulation range of the pause (parameter 11 in **Figure 7E**) and the time to lowest firing frequency of type B IPns during the pause (parameter 12 in **Figure 7E**) with respect to CS presentation. The time-intensity components of the neuronal distribution allowed us to determinate the relative time dispersion patterns between type B neural commands and the eyelid kinematics (see the green and brown circular sectors in **Figure 7D**). According to data shown in **Figures 7B,D**, the time to lowest firing rate of type B IPns always lagged the beginning of the CRs [green arrows, mean timing $\overline{T}s = 70.11 \pm 2.80$ ms, green circular sector, mean timing $\overline{T} = 71.26 \pm 3.03$ ms, (mean $\pm$ SEM); brown arrows, mean

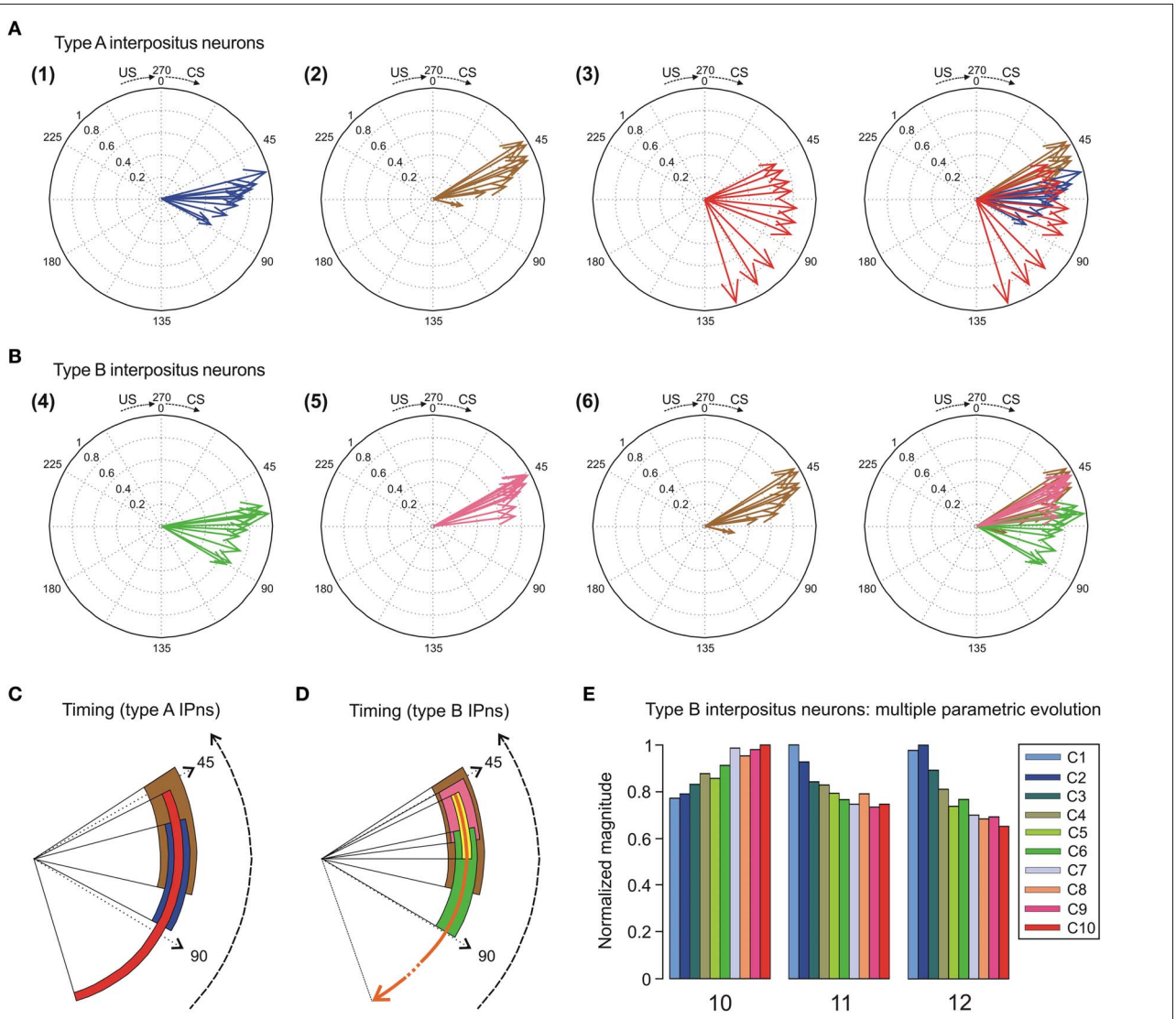

FIGURE 7 | Compass plots of the time-intensity distributions across conditioning sessions. (A) In this representation, the parametric timing-intensity distributions are shown as **(1)** time to peak firing rate of type A interpositus neurons (IPns) vs. peak firing frequency of type A IPns, blue arrows; and **(2)** time to conditioned response (CR) onset vs. percentage of CRs, brown arrows. In addition, the time delay-strength distribution is shown as **(3)** time delay in coupling vs. maximum linear correlation coefficient between type A IPns firing rate and eyelid position, red arrows. These parameters were plotted using the circular statistics for an inter-stimulus interval (ISI, CS–US interval) of 270 ms. The 10 colored arrows (10 conditioning sessions, C1–C10) in each circle illustrate the circular dispersion of the angular datasets represented. **(B)** The compass plot representation for type B IPns, but according to the following distributions: **(4)** time to lowest firing rate of type B IPns during the pause [parameter 12 in **(E)**] vs. the lowest firing frequency during the pause, green arrows; and **(5)** time to peak firing rate of type B IPns in the modulation range of the pause [parameter 11 in **(E)**] vs. peak firing frequency of type B IPns in the modulation range of the pause [parameter 10 in **(E)**], pink arrows. The distribution **(6)** is the same one that the **(2)**. **(C,D)** Interactions between parametric timing information and time delay in coupling between the firing rate of the types A and B IPns and the eyelid position response. The colored circular sectors in **(C)** and **(D)** illustrate the time-dispersion range of the data distributions represented in the panels **(A)** and **(B)**, respectively. In **(D)**, the yellow circular sector indicates the temporal range of beginning of the pause, and the orange circular arrow represents the duration of the pause (including the temporal ranges for the yellow and green circular sectors) across conditioning sessions. **(E)** Multiple parametric evolutions of timing and kinetic variables (parameters 10, 11, and 12) for type B IPns across conditioning sessions.

timing $\overline{\mathrm{Ts}} = 52.62 \pm 1.89$ ms, brown circular sector, $\overline{\mathrm{T}} = 56.92 \pm 2.17$ ms, (mean $\pm$ SEM)]. Finally, we illustrate the multiple parametric evolutions (**Figure 7E**) including the above kinetic (parameter 10) and timing (parameter 11 and 12) variables across conditioning sessions. Interestingly, parameter 10 did not change significantly [One-Way ANOVA F-tests, $F_{(9,45,98)} = 1.37$, $P > 0.05$] and the parameters 11 and 12 [One-Way ANOVA F-tests, $F_{(9,45,98)} = 5.92$, $P < 0.01$; $F_{(9,45,98)} = 2.07$, $P < 0.05$, respectively] decreased across conditioning sessions. However, the time to peak firing rate of type B IPns in the modulation range of the pause with respect to the beginning of the CR (not with respect to CS presentation) did not change significantly [One-Way ANOVA F-tests, $F_{(9,45,98)} = 1.82$, $P > 0.05$] across conditioning.

Table 1 | The time-intensity dispersion indices corresponding to the circular distributions of the datasets across conditioning sessions.

Time-intensity dispersion indices (ISI = 270 ms)								
Mean angle ($\overline{\Omega}$s, in radians)		**Mean timing ($\overline{T}$s, in milliseconds)**		**Mean radius of the centroid ($\overline{C}$s)**		**Circular kurtosis index (ρs)**		**Time–intensity dispersion index (σs)**	
$\overline{\Omega}s_{IPn-A}$	1.5741	$\overline{T}s_{IPn-A}$	67.6409	$\overline{C}s_{IPn-A}$	0.0717	ρs_{IPn-A}	0.8875	σs_{IPn-A}	10.9241
$\overline{\Omega}s_{CR}$	1.2246	$\overline{T}s_{CR}$	52.6245	$\overline{C}s_{CR}$	0.0701	ρs_{CR}	0.8532	σs_{CR}	14.9448
$\overline{\Omega}s0$	1.8498	$\overline{T}s0$	79.4898	$\overline{C}s0$	0.0732	$\rho s0$	0.5092	$\sigma s0$	45.8350
$\overline{\Omega}s_{IPn-B1}$	1.6315	$\overline{T}s_{IPn-B1}$	70.1083	$\overline{C}s_{IPn-B1}$	0.0802	ρs_{IPn-B1}	0.8792	σs_{IPn-B1}	9.3971
$\overline{\Omega}s_{IPn-B2}$	1.1662	$\overline{T}s_{IPn-B2}$	50.1144	$\overline{C}s_{IPn-B2}$	0.0888	ρs_{IPn-B2}	0.9718	σs_{IPn-B2}	1.7861

*The duration of the inter-stimulus interval (ISI) is 270 ms. Here, the intensity/strength components have been normalized in accordance with their maximum value across conditioning sessions. These quantitative results are in correspondence with the circles in **Figures 7A,B**. The indices X_{IPn-A}, X_{CR}, $X0$, X_{IPn-B1}, and X_{IPn-B2} (where $X = \overline{\Omega}s$, $\overline{T}s$, $\overline{C}s$, ρs, or σs) correspond to the distributions (1), (2), (3), (4), and (5), respectively (see the legend of **Figure 7**).*

Table 2 | The time dispersion indices corresponding to the circular distributions of the datasets across conditioning sessions.

Time-dispersion indices (isi = 270 ms)								
Mean angle ($\overline{\Omega}$, in radians)		**Mean timing ($\overline{T}$, in milliseconds)**		**Mean radius of the centroid ($\overline{C}$)**		**Circular kurtosis index (ρ)**		**Time–dispersion index (σ)**	
$\overline{\Omega}_{IPn-A}$	1.6228	$\overline{T}_{IPn-A}$	69.7354	$\overline{C}_{IPn-A}$	0.0972	ρ_{IPn-A}	0.8910	σ_{IPn-A}	5.7710
$\overline{\Omega}_{CR}$	1.3245	$\overline{T}_{CR}$	56.9157	$\overline{C}_{CR}$	0.0966	ρ_{CR}	0.8681	σ_{CR}	7.0724
$\overline{\Omega}0$	1.8031	$\overline{T}0$	77.4804	$\overline{C}0$	0.0858	$\rho 0$	0.5181	$\sigma 0$	32.7060
$\overline{\Omega}_{IPn-B1}$	1.6583	$\overline{T}_{IPn-B1}$	71.2615	$\overline{C}_{IPn-B1}$	0.0969	ρ_{IPn-B1}	0.8799	σ_{IPn-B1}	6.3922
$\overline{\Omega}_{IPn-B2}$	1.1757	$\overline{T}_{IPn-B2}$	50.5213	$\overline{C}_{IPn-B2}$	0.0993	ρ_{IPn-B2}	0.9720	σ_{IPn-B2}	1.4220

*The duration of the inter-stimulus interval (ISI) is 270 ms. Here, the matrix of intensity/strength components has been substituted by a matrix of those to fit their values to the unitary circle. These quantitative results are in correspondence with the circular sectors in **Figures 7C,D**. The indices X_{IPn-A}, X_{CR}, $X0$, X_{IPn-B1}, and X_{IPn-B2} (where $X = \overline{\Omega}$, $\overline{T}$, $\overline{C}$, ρ, or σ) correspond to the distributions (1), (2), (3), (4), and (5), respectively (see the legend of **Figure 7**).*

In **Tables 1** and **2**, we summarize the results including the statistical parameters that enabled us to describe the different patterns of dispersions for our dataset distributions. Notice the difference in the values of the dispersion indices between the time-intensity distributions (**Table 1**) and time distributions (**Table 2**) of the datasets. For the reports in **Table 1**, the intensity/strength components for all the data distributions have been normalized previously in accord with their maximum value across conditioning (see **Figures 7A,B**). In contrast, the matrix of intensity/strength components for the results in **Table 2** has been substituted by a matrix of those to fit their values to the unitary circle (see **Figures 7C,D**). In fact, we calculated the different dispersion indices ($\overline{\Omega}$s, $\overline{T}$s, $\overline{C}$s, ρs, and σs, see **Table 1**; and $\overline{\Omega}$, $\overline{T}$, $\overline{C}$, ρ, and σ, see **Table 2**) to reveal the true parametric timing-intensity and time delay-strength dispersion patterns between eyelid kinematic and either type A or type B IPns activities in the different temporal domains (inter-trials dispersion of the same block, inter-blocks dispersion of the same session, and inter-sessions dispersion along the process)—i.e., the time–intensity contributions (at least in the circular statistical sense) of the different neuronal centers (cerebellar interpositus and facial nuclei) participating in this associative learning process. In accordance with the above results, we analyzed the mean values of angle, timing, radius of centroid, circular kurtosis, and dispersion index for all the circular patterns [form (1) to (5) in **Figure 7**] using the *von Mises distribution* (the circular analog of the normal distribution). Thus, we could reject the Watson hypothesis test [e.g., explores whether $\overline{T}(i)$ has the same mean for the different circular distributions of the timing data along the different training blocks, sessions, and for all the subjects] and to verify the temporal order of our physiological data. Here, the mean timing to peak firing rate of type A IPn was always bigger than the mean timing to CR onset respect to CS presentation, and the same for the time to lowest firing frequency of type B IPn. It could thus be concluded that the firing activities of IPns and their temporal dynamics may be related more with the proper performance of ongoing CRs (including the proper time-intensity dispersion patterns) than with their generation and/or initiation (**Figures 7A–D** and **Tables 1–2**).

DISCUSSION

Seminal electrophysiological recordings of putative cerebellar nuclei units made in behaving rabbits revealed that eyeblink-related neurons are mostly located in the rostral aspects of the interpositus nucleus (McCormick and Thompson, 1984; Berthier and Moore, 1990). In contrast, eyelid-related neurons in behaving cats seem to occupy more-caudal locations—i.e., in the rostral part of the posterior interpositus nucleus (Gruart and Delgado-García, 1994; Gruart et al., 2000a; Delgado-García and Gruart, 2002). Nevertheless, neurons firing in response to facial mechanoreceptor activation were also observed in both the rostral interpositus and dentate nuclei (Gruart and Delgado-García, 1994). In agreement with recordings made in behaving monkeys (Van Kan et al., 1993), a detailed mapping of the three cerebellar

nuclei in alert behaving cats indicates that neurons related to eyelid movements are preferentially located in the rostro-dorsolateral aspect of the posterior interpositus nucleus (Gruart et al., 2000a). In this regard, recent data collected from mice (Porras-García et al., 2010) and rats (Morcuende et al., 2002; Chen and Evinger, 2006) also located eyeblink-related neurons in the dorsolateral hump and in the posterior interpositus nucleus, but not in the anterior subdivision of the nucleus. To date, there is no better explanation for these disparities in the location of eyeblink-related neurons than the possible neural differences within different species and/or the different experimental procedures used for their recording and identification. Nonetheless, we concentrated here on functional relationships and putative roles of antidromically identified type A and B IPns and OO MNs.

AN AGONIST–ANTAGONIST NUCLEAR SYSTEM CONTROLLING EYELID BIOMECHANICS DURING MOTOR LEARNING

We have analyzed here the firing activities of type B posterior IPns, and compared them with those of by OO Mns and type A posterior IPns (Trigo et al., 1999; Gruart et al., 2000a; Delgado-García and Gruart, 2002; Sánchez-Campusano et al., 2007, 2009, 2011b). The analytical-experimental approach we developed (cumulative neural integration method, time-intensity dispersion models, multiple parametric evolutions, and raster and 3D representations in the time and frequency domains) enabled us to determine three lines of functional evidence that demonstrate the antagonistic properties of the two types (types A and B) of posterior IPn identified in alert behaving cats during classical eyeblink conditioning, using a delay paradigm.

First, a pause in the firing activity of type B IPns during the active downward displacement of the upper eyelid is not consistent with a role of these neurons in the active modulation of the firing rate of the OO Mns. This decreased activity would reduce the excitatory drive to facial Mns through the contralateral red nucleus (Morcuende et al., 2002; Chen and Evinger, 2006). In contrast, this active modulatory role on the final motor pathway of conditioned eyelid responses during the downward displacement has been described for type A IPns (Gruart et al., 2000a; Delgado-García and Gruart, 2002; Sánchez-Campusano et al., 2007, 2009). According to the present results, the mean peak firing rate of type A IPns increased across conditioning whilst the time interval between CR onset and their maximal discharge decreased, causing a drop in the correlation between the instantaneous discharge of type A IPns and conditioned eyelid responses (see **Figure 5C**). In addition, the increase in firing rate, in association with the decrease in its mean time of occurrence, caused a drop in the coefficient of correlation between the firing rate of type A IPns and conditioned eyelid responses (see the red arrows in **Figure 7A**). As a result, the time to maximum correlation (i.e., the time delay) always lagged the beginning of the CR (see the red and brown circular sectors in **Figure 7C**). Furthermore, our previous analysis of dynamic association (Sánchez-Campusano et al., 2009) allowed us to determine an asymmetric, non-linear, and non-unidirectional coupling between type A IPns and OO EMG recordings—i.e., type A IPs do not directly encode eyelid kinematics. These results could be explained if we assume that there is an indirect reinforcement and/or modulatory effect of type A IPns on OO Mns. In this sense, type A cerebellar posterior IPns could contribute to facilitating a quick repolarization of OO Mns, reinforcing their tonic firing during the active contraction of the OO muscle and, therefore, during the active eyelid downward displacements of the upper eyelid. In turn, the discharge rate of the OO Mns increased progressively across the learning process, with a relative refractory period (minimum interspike time interval) that decreased progressively in the CS–US interval (see **Figure 5D**). This inference is supported by the experimental fact that the total number of spikes generated by these facial Mns during the CS–US interval increased across the learning process in relation to the increased number of muscular action potentials from OO EMG activity and, therefore, with the progressive increase in the amplitude of the corresponding conditioned eyelid responses (see **Figure 5E**). Thus, an OO Mn pattern of discharge that correlated significantly with eyelid position (Trigo et al., 1999; Sánchez-Campusano et al., 2007) and OO EMG activity (Sánchez-Campusano et al., 2009, 2011b) during CRs was obtained in all the conditioning sessions.

Second, the integrated neural activity of type B IPns did not show oscillatory properties—e.g., a saturation of the cumulative integrated pattern (**Figures 4C,E**) determined by the sustained pause in their firing frequency in the CS–US interval (the green profiles in **Figure 3B**). In contrast, the neural nature of the 50 ms mean period of oscillation characteristic of eyelid kinematics (see the 20 Hz spectral component in **Figures 6C,F**) can be suitably explained by the oscillatory properties (at the same fundamental frequency and period) of the relative variation functions of both type A IPns and OO Mns integrated neural activities (**Figures 4B,D**) during this associative learning process. Furthermore, and in accordance with the above results (**Figure 4B**), the reinforcing-modulating role of cerebellar circuits of ongoing conditioned eyelid responses is highly dependent on their adequate phase modulation with respect to intrinsic facial Mns oscillatory properties. Thus, type A IPns could be considered to behave as a neuronal phase-modulating device supporting OO Mns firing during learned eyelid movements (Sánchez-Campusano et al., 2007).

Third, the timing analysis of the cessation of firing activity of the type B IPns allowed us to demonstrate that their time to peak firing rate in the modulation range of the pause (see green arrow in **Figures 4C,E**) with respect to the beginning of the CRs (see brown circle in **Figure 4E**) did not change significantly [One-Way ANOVA F-tests, $F_{(9,45,98)} = 1.82$, $P > 0.05$] across conditioning sessions. In turn, the mean values of the latency to peak firing rate of type A IPns during the evolution across all the conditioning sessions were always located within the temporal range of the pause of type B IPn firing rate, but always after the onset of CRs. The mean delays between the critical points (peak firing rate of type A IPns, lowest firing rate of type B IPns) and the mean time to CR onset were $\Delta t \approx 20$ ms during the first days of conditioning (e.g., for the sessions C3–C6, $\Delta t = b$–c in **Figures 5A** and **6A,B**) and $\Delta t \approx 16$ ms at the asymptotic level of acquisition of this associative learning test (i.e., session C10, **Figure 4E**). Moreover, in accordance with parameters 2 (**Figure 5C**) and 8 (**Figure 5E**), the mean delay Δt along the conditioning sessions was 12.81 $\pm$ 0.28 ms (mean $\pm$ SEM). However, the main outcome of this

timing analysis was that the latency to the initial modulation of the pause in type B IPns was always smaller than the latency to the initial modulation of the burst in type A IPns, just the time necessary for the amplitudes of the two firing frequencies (for types A and B IPns) to reach the critical points (peak and lowest firing frequencies, **Figures 4E**, **5A**, and **6A,B**) of their antagonistic behavior simultaneously. This inference is supported by the experimental fact that the two types of neuron (types A and B IPns) have similar antidromic latencies (latency in the range 0.5–1.2 ms, see Gruart et al., 2000a; Jiménez-Díaz et al., 2004; Sánchez-Campusano et al., 2007) after red nucleus stimulation, and therefore, an action potential of these neurons should affect EMG activity of the involved eyelid muscles 5–6 ms later, combining an assumption of equal conduction velocities for the interpositus nucleus projection to the red nucleus and for the red nucleus projection to the motoneuronal system with the 2 ms latency of EMG activity after motoneuron discharge (Pellegrini et al., 1995; Chen and Evinger, 2006). Finally, we can suggest that the two types of simultaneous action (facilitation and disfacilitation) are in accordance with a dominant role of cerebellar circuits for the facilitation of flexor responses. Thus, type A IPns fire during active eyelid downward displacements—i.e., during the active contraction of the OO muscle (a facilitation of OO Mns). In contrast, type B IPns present a high tonic rate when the eyelids are wide open, and stop firing during any active downward displacement of the upper eyelid—i.e., a disfacilitation of LPS Mns.

Although the contribution of cerebellar circuits to classical eyeblink conditioning is still controversial (Llinás and Welsh, 1993; Mauk, 1997; Seidler et al., 2002; Koekkoek et al., 2003; Welsh et al., 2005; Delgado-García and Gruart, 2006; Kreider and Mauk, 2010), we can suggest from an anatomo-functional point of view that type B IPns project, by the ascending limb of the posterior peduncle, not only to the red nucleus and to the mesencephalic oculomotor area, but also to, among other structures, the perioculomotor area (Porter et al., 1989; Gonzalo-Ruiz and Leichnetz, 1990; Gonzalo-Ruiz et al., 2004)—that is, more or less directly onto LPS Mns (Gruart and Delgado-García, 1994). In addition, it could be suggested that type B IPns play a facilitative role in the antagonistic action of the LPS muscle. In this sense, our experimental-analytical approach to the study of cerebellar function takes into account the neural (types A and B IPns to modulate the OO and LPS Mns, respectively) and biomechanical (OO and LPS muscles) elements of a double activation–deactivation system controlling eyelid kinematics (eyelid position, velocity, and acceleration profiles) during motor learning.

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Developmental disorders of the midbrain and hindbrain

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Malformations of the midbrain (MB) and hindbrain (HB) have become topics of considerable interest in the neurology and neuroscience literature in recent years. The combined advances of imaging and molecular biology have improved analyses of structures in these areas of the central nervous system, while advances in genetics have made it clear that malformations of these structures are often associated with dysfunction or malformation of other organ systems. This review focuses upon the importance of communication between clinical researchers and basic scientists in the advancement of knowledge of this group of disorders. Disorders of anteroposterior (AP) patterning, cerebellar hypoplasias, disorders associated with defects of the pial limiting membrane (cobblestone cortex), disorders of the Reelin pathway, and disorders of the primary cilium/basal body organelle (molar tooth malformations) are the main focus of the review.

Keywords: midbrain, hindbrain, cerebellum, malformations

INTRODUCTION

For many years, anomalies of the cerebellum and brain stem were poorly reported in the scientific literature. The cerebellum was believed to have a minor role in brain function, while the brain stem was difficult to remove intact at autopsy and difficult to section. Radiologic analysis of these structures by pneumography, angiography, and X-ray computed tomography was poor. Recently, however, advances in developmental genetics, neurobiology, molecular biology, and neuroimaging have led to better understanding of developmental disorders of the embryonic midbrain (MB) and hindbrain (HB), which grow into the adult brainstem and cerebellum (Barkovich et al., 2007, 2009). Although malformations of the brainstem and cerebellum may be the only recognized abnormality in individuals with mental retardation or autism (Soto-Ares et al., 2003; Courchesne et al., 2005), they are more commonly identified in patients with malformations of the cerebrum. Among the most common of these are lissencephalies (Ross et al., 2001; Lecourtois et al., 2010), so-called "cobblestone malformations" of the cortex (formerly known as lissencephaly type II) resulting from defects in the pial limiting membrane (van Reeuwijk et al., 2006; Clement et al., 2008; Hewitt, 2009), anomalies of the cerebral commissures (Barkovich et al., 2007), and disorders of primary cilia function that include additional ocular, renal, hepatic, and limb bud anomalies (Lancaster et al., 2011; Sang et al., 2011).

The number and complexity of recognized malformations of the brainstem and cerebellum has been steadily increasing. These disorders were recently extensively reviewed and classified (Barkovich et al., 2009). This review will highlight a few MB-HB malformations and emphasize how knowledge of basic research in embryology, genetics, and cellular and molecular biology of the developing brain can be of importance in recognizing, understanding, and classifying these anomalies in humans.

MALFORMATIONS SECONDARY TO EARLY PATTERNING DEFECTS

Malformations of the MB or HB secondary to defects in anteroposterior (AP) or dorsoventral (DV) patterning were nearly impossible to identify in neurology patients until magnetic resonance imaging became a commonly used tool in clinical diagnosis. The ability to acquire high resolution, high contrast, distortion-free images in sagittal and coronal planes allowed accurate gross assessment of MB-HB structures for the first time. However, the structures within the MB-HB are small and move with cardiac pulsations; therefore, physicians were slow to recognize subtle distortions in their structure and recognize their importance in developmental disorders. As a consequence, physicians have only recently begun to look for subtle variations in them. Only in the past 10 years have these malformations been fairly consistently identified and associated with normal developmental processes and their derangements (Jen et al., 2004; Bednarek et al., 2005; Moog et al., 2005; Sicotte et al., 2006; Barkovich et al., 2007; Barth et al., 2007; Jissendi-Tchofo et al., 2009).

DISORDERS OF A-P PATTERNING

The easiest malformations to identify are those due to disturbed AP patterning, particularly at the MB-HB junction, which is defined in mice (and presumably in humans) by a balance of Otx2 and Gbx2 signaling that defines the position of the isthmus organizer (IsO) (Wassef and Joyner, 1997; Millet et al., 1999; Chizhikov and Millen, 2003). The IsO ultimately defines the posterior limit of the MB and the anterior limit of the cerebellum (Chizhikov and Millen, 2003) (**Figure 1A**). The combination of a shortened MB and elongated pons associated with an enlarged anterior vermis in humans **(Figure 1B)**, therefore, presumably results from rostral displacement of the IsO, with loss of MB and gain of R1 [from which the cerebellum forms,

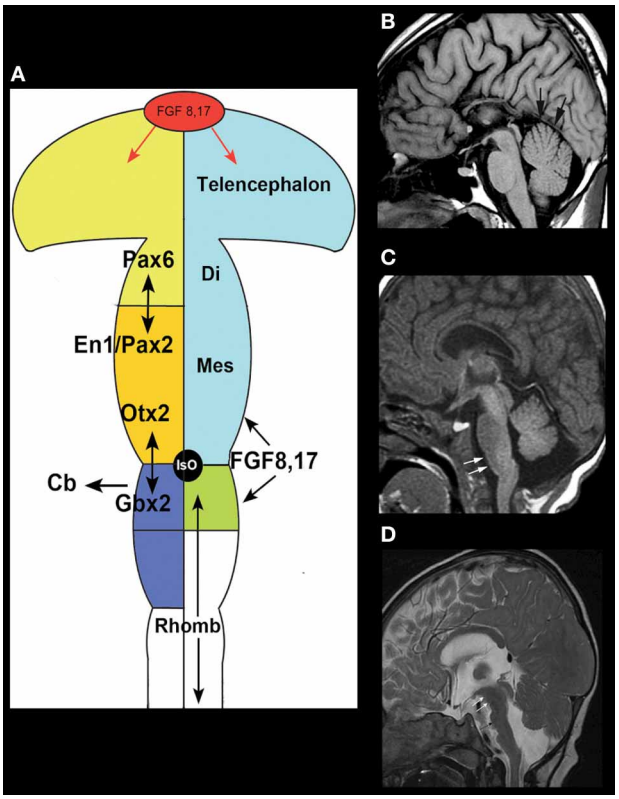

FIGURE 1 | Disorders of AP patterning in the brain stem. **(A)** Anteroposterior patterning and the Isthmus Organizer. Regionalization of the brain starts with the formation of patterning centers that secrete signaling molecules such as the fibroblast growth factors (FGFs). Fgf8 and Fgr17 are important signaling molecules at both the anterior forebrain and the MB-HB junction. In the forebrain, it helps to direct formation of the prefrontal cortex and other rostral structures by inducing cells to secrete the transcription factor Pax6. At the MB-HB junction, the patterning center known as the *isthmus organizer* (IsO) is localized and induced to secrete Fgf8 and Fgf17 by the interaction of transcription factor Gbx2 from the rhombencephalon and Otx2 from the caudal mesencephalon. The secretion of Fgf8 and Fgf17 then induces further changes crucial to formation of the MB-HB junction and the formation of the cerebellum. The junction of the diencephalon (di) and mesencephalon (mes) is directed by the interaction of Pax6 from the diencephalon and En1/Pax2 from the rostral mesencephalon. Repression of Otx2 expression by FGF8 induces Gbx2 formation to establish the location of the MB-HB junction and can affect cerebellar formation, as the cerebellum forms from the most rostral portion of the HB. Similarly, alterations of Pax6 or En1/Pax2 will alter the location of the diencephalic-mesencephalic junction. (Adapted from Barkovich and Raybaud, 2012). **(B)** Sagittal T1 weighted image shows a short MB, long pons, and large superior vermis (black arrows), suggesting an abnormality of anteroposterior patterning with rostral misplacement of the Isthmus Organizer. In addition, the patient has agenesis of the corpus callosum. **(C)** Sagittal T1 weighted image shows a slightly small pons and a short, thick medulla (white arrows) with an abnormal pontomedullary transition. This is postulated to result from mixed gains and losses of rhombomere expression in the developing rhombencephalon or potentially a segmental shift of rhombomeres. **(D)** Sagittal T2 weighted image shows a very elongated MB (white arrows) with small, short pons (black arrow), and small cerebellar vermis, suggesting caudal displacement of the Isthmus Organizer due to abnormal anterioposterior patterning from overexpression of Otx2.

with the vermis deriving from the most rostral portion (Broccoli et al., 1999; Chizhikov and Millen, 2003)]; this malformation is presumed to result from GBX2 predominance over OTX2 and consequent rostral malpositioning of the IsO (Chizhikov and Millen, 2003; Barkovich et al., 2009). This finding has been described in Opitz G/BBB syndrome (OS), an X-linked form of which is caused by loss of function mutations of the *MID1* gene (Quaderi et al., 1997). MID1 plays a role in the ubiquitin-specific regulation of the microtubule associated catalytic subunit of protein phosphatase 1Ac (Aranda-Orgillés et al., 2008). Its role in the pathogenesis of the disease is not clear. Patients show variable clinical signs and symptoms affecting multiple organ systems. Imaging shows hypoplasia of the anterior cerebellar vermis (Pinson et al., 2004; Fontanella et al., 2008). *Mid1*-null mice show motor coordination defects and procedural learning impairments. Of note, in addition to cerebellar vermian hypoplasia, these mice show shortening of the posterior dorsal MB, rostralization of the MB-HB, and down-regulation of Fgf17, a key transcription factor in the region (Lancioni et al., 2010). This is another area in which applying learning from human disease is helping to understand development in the MB-HB region.

Shortening and thickening of the medulla (**Figure 1C**) (Barkovich et al., 2009) is postulated to result from mixed gains and losses of the eight rhombomeric segments within the pons and medulla or a segmental shift of rhombomeres; rhombomeres may be absent or they may be misexpressed, taking on characteristics of other rhombomeres. Such anomalies give the brain stem an abnormal shape (**Figures 1C,D**). Similar abnormalities result from murine embryo exposure to retinoic acid, which causes a dose-dependent anterior to posterior transformation of cell fate in which the HB is expanded at the expense of the MB and forebrain (Lumsden, 2004). Lesser changes in gradients of retinoic acid or other regionalizing molecules could result in transformations of the middle rhombomeres from pontine to medullary fate. Such changes are difficult to assess with current imaging techniques but may become possible as higher resolution/high field strength MR scanners are developed, along with better tractography programs.

DISORDERS WITH CEREBELLAR HYPOPLASIA

Just as rostral displacement of the MB-HB junction is expected to increase the size of rhombomere 1, caudal displacement of the IsO [presumably due to increased OTX2 (Broccoli et al., 1999)] would be expected to elongate the MB, and shorten the pons (particularly the R1 segment); the expected result would be cerebellar hypoplasia, particularly affecting the vermis (which is formed from the most rostral aspect of R1) (Chizhikov and Millen, 2003). Indeed, in the few cases observed clinically with an elongated MB and small pons, the cerebellum has always been small (**Figure 1D**). Cerebellar hypoplasias are common findings in autopsy studies and in clinical neuroimaging and have many causes (Barkovich et al., 2009). Although cerebellar hypoplasia may be an isolated finding, it is usually associated with other anomalies, which may be either supratentorial or infratentorial. A few examples follow.

The *Dandy–Walker malformation* and related disorders (cerebellar hypoplasia, mega cisterna magna, and Blake pouch cysts) are composed of a grouping of abnormalities of the cerebellum, its surrounding mesenchyme, and sometimes cerebral structures; this variable combination of features has generated considerable confusion and controversy (Raybaud, 1982; Raimondi et al., 1984; Barkovich et al., 1989; Tortori-Donati et al., 1996). The *Dandy–Walker malformation*, as initially defined, consists of an enlarged posterior fossa with a high position of the tentorium cerebelli, counterclockwise rotation and hypoplasia of the cerebellar vermis, and a dilated, cystic-appearing fourth ventricle that fills nearly the entire posterior fossa, presumably due to cyst-like expansion of the fourth ventricle (**Figure 2A**) (Hart et al., 1972; Raimondi et al., 1984). The cerebellar hemispheres are usually small and corpus callosal anomalies are found in as many as 20% of affected individuals (Barkovich et al., 1989). Significant variation in cerebellum, brain stem, surrounding CSF spaces, and associated supratentioral anomalies of all degrees may be found, however, in the malformation complex. Indeed, considerable variation can be seen in families with the *same* genetic mutation; the phenotype ranges from mild vermian hypoplasia to mega cisterna magna to varying severities of true Dandy–Walker malformation (Grinberg et al., 2004; Aldinger et al., 2009; Blank et al., 2011). It is noteworthy that *FOXC1,* which has been shown to cause this malformation complex in families, is expressed only in the mesenchyme overlying the cerebellum and not in the cerebellum itself (Aldinger et al., 2009). Similar ranges of posterior fossa anomalies have been described with deletion of 3q24 (loss of *ZIC1–ZIC4*) (Grinberg and Millen, 2005), duplication of 9p (Melaragno et al., 1992; Cazorla Calleja et al., 2003; Chen et al., 2005), deletion of 13q2 (McCormack et al., 2003; Ballarati et al., 2007), and deletion of 2q36.1 [which contains the PAX3 gene, strongly expressed in the developing cerebellum (Jalali et al., 2008)], as well as in neurocutaneous melanosis [a dysplasia of the leptomeninges that is most severe in the basal meninges around the brain stem and cerebellum (Narayanan et al., 1987; Barkovich et al., 1994; Acosta Jr., et al., 2005)]. Of note, MB-HB hypoplasia is only seen in neurocutaneous melanosis when melanosis is present in the meninges surrounding the brain stem and cerebellum, supporting the hypothesis that the developing leptomeninges have significant effects upon MB-HB development (Aldinger et al., 2009). Based upon all of these observations, it may be suggested that (1) the surrounding mesenchyme affects growth of the developing cerebellum during embryogenesis, and (2) mutations resulting in dysgenesis of both the cerebellum and its overlying mesenchyme are likely to be necessary for the entire Dandy–Walker malformation complex to form, with less severe dysgenesis resulting in malformations such as isolated cerebellar hypoplasia or isolated dysgenesis of the surrounding mesenchyme. In light of this information, it was suggested that the Dandy–Walker malformation be considered in the group of *mesenchymal-neuroepithelial signaling defects* (Barkovich et al., 2009). From this perspective, it follows that retrocerebellar arachnoid cysts and enlargement of the cisterna magna (called mega cisterna magna, an enlarged posterior fossa secondary to an enlarged cisterna magna, but a normal cerebellar vermis and fourth ventricle, **Figure 2B**) are a part of the Dandy–Walker spectrum from an embryogenesis perspective. In addition, the so-called persistent Blake pouch cyst, where the ependymal wall of the fourth ventricle extends through the foramen of Magendie and upwardly rotates a normal cerebellar vermis (**Figure 2C**), is sometimes considered a part of the spectrum. However, in the absence of mass effect (causing hydrocephalus) or associated cerebral/cerebellar dysgenesis, none of these findings seem to be of clinical significance; neurological and cognitive development seem to be related, instead, to the level of control of hydrocephalus, to the extent of associated supratentorial anomalies (Golden et al., 1987; Maria et al., 1987; Bindal et al., 1990–1991), and to the degree of cerebellar dysgenesis, manifested as lobulation of the vermis; normal lobulation is associated with good intellectual outcome whereas some have found that abnormal vermian lobulation is associated with poor intellectual outcome (Boddaert et al., 2003; Klein et al., 2003). The molecular

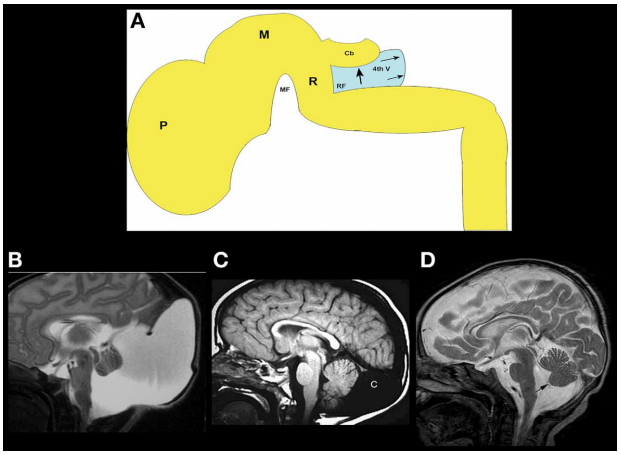

FIGURE 2 | The concept and range of cerebellum/posterior fossa disorders in the "Dandy–Walker spectrum." (A) Illustration of developing fourth ventricle/cerebellum and the impact of impaired egress of CSF. During normal development, the wall fourth ventricle normally thins, and the foramen of Magendie forms, in the midline. If the leptomeninges are abnormal, the cerebellum may be small and the outflow foramina of the fourth ventricle may not form. The fourth ventricle expands posteriorly (small black arrows) and superiorly, pushing the small cerebellar vermis (Cb) counterclockwise (large black arrow); the posterior fossa then enlarges. This combination of findings creates the classic Dandy–Walker malformation. Small black PF signifies pontine flexure, small black MF signifies mesencephalic flesure, large black P signifies prosencephalon, large black M signifies mesencephalon, large black R signifies rhombencephalon. **(B)** Classic Dandy–Walker malformation. Sagittal T2 weighted image shows the classic appearance with a small vermis (black arrow), rotated counterclockwise with abnormal foliation. The surrounding CSF spaces are markedly enlarged with abnormally enlarged posterior fossa and elevation of the tentorium cerebelli and the torcular Herophili. **(C)** Mega cisterna magna is a condition in which a collection of CSF in an enlarged cisterna magna (**C**) expands the posterior fossa but the midbrain and hindbrain are normal. It is seen in patients with gene mutations that, in siblings, cause the classic Dandy–Walker malformation. **(D)** Blake pouch cyst is a condition in which the ependymal wall of the fourth ventricle stretches out through the foramen of Magendie and causes enlargement of the foramen with mild rotation of the vermis (black arrow). It is considered by some to be an incidental finding and by others to be a mild form of the Dandy–Walker malformation.

biologic pathways involved in these disorders have not yet been identified.

MB-HB ANOMALIES ASSOCIATED WITH CEREBRAL ANOMALIES

MB-HB anomalies are often found in association with supratentorial brain anomalies. Often, these are the result of similar processes occurring in both the forebrain and HB. For example, the so-called "*cobblestone malformations*" or "*dystroglycanopathies*" are disorders that are caused by impaired linkage of the endfeet of radial glial cells with the pial limiting membrane **(Figure 3A)**, in many cases because of decreased O-glycosylation of a-dystroglycan, which impairs its binding to Laminin-2 in the basal lamina of that membrane (Saito et al., 2006; Godfrey et al., 2007; Clement et al., 2008; Li et al., 2008; Hewitt, 2009; Chan et al., 2010; Chiang et al., 2011). [These cerebral disorders were formerly called "lissencephaly type II" or "cobblestone lissencephaly." That nomenclature resulted in considerable confusion with lissencephalies caused by undermigration of neurons destined for the cerebral cortex. As a result, the term cobblestone malformation is now preferred (Barkovich et al., 2012)]. Other causes of abnormal linkage include altered laminin deposition (Ackroyd et al., 2011), mutation of G protein-coupled receptor 56 (Gpr56) or its receptor collagen type III (Luo et al., 2011), and mutation of the ubiquitous basement membrane protein collagen type IV alpha 1 (Labelle-Dumais et al., 2011). The result of these abnormal linkages is that some migrating neurons leave the radial glial fiber before reaching their intended cortical layer and other neurons overmigrate through "gaps" form in the pial limiting membrane into the subarachnoid space **(Figure 3A)** (Martin, 2005). The resulting cerebral cortex is composed of radially oriented clumps of disoriented neurons, the subarachnoid space is filled with ectopic neurons that have overmigrated, and multiple nodules of undermigrated heterotopic neurons lie in the subcortical white matter (Friede, 1989; Norman et al., 1995; Haltia et al., 1997). Similar phenomena of gaps in basal lamina with over- and undermigrated neurons may be seen in the retina and the cerebellum (Friede, 1989; Norman et al., 1995; Haltia et al., 1997). Abnormal linkages using the same molecular structures are found in skeletal muscle, with the result being that many affected patients also have congenital muscular dystrophy (Moore et al., 2002; Martin, 2005; Kanagawa and Toda, 2006). In the cerebellum, the leptomeninges are of considerable importance for the normal migration of granule cells (Zarbalis et al., 2007; Koirala et al., 2009). Mice with dystroglycanopathies show widespread discontinuities in the pial basement membrane

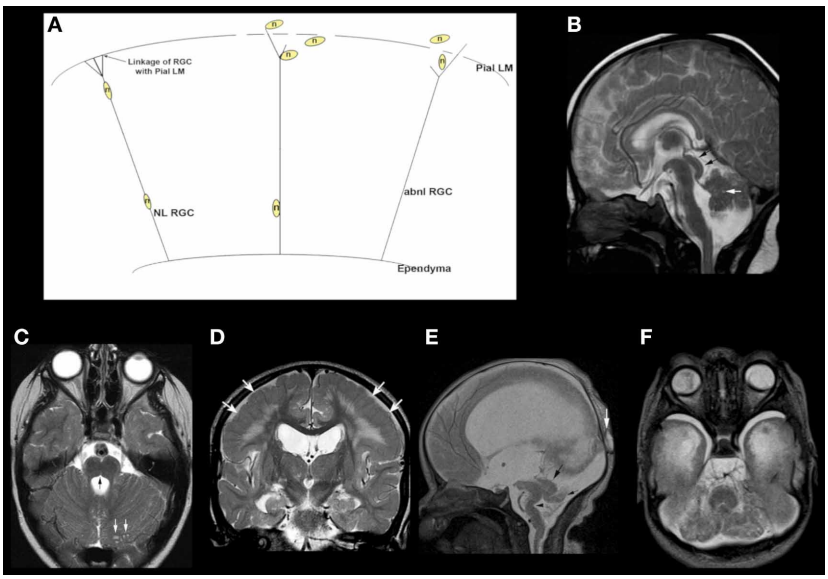

FIGURE 3 | Array of findings in the midbrain and hindbrain of cobblestone malformations (formerly called Lissencephaly type II). Diagram **(A)** shows neuron (n) guided by normal radial glial cell (NL RGC) on the left, coursing from ependyma to an intact pial limiting membrane (Pial LM), where it attaches via a bridge made by beta dystroglycan, alpha dystroglycan, or GPR56, which attach to laminin-2 or collagen IV in the Pial LM. In the center and on the right, gaps are seen in the Pial LM; the RGCs do not attach properly due to defects of alpha dystroglycan or GPR56 in the leading process of the RGC, to laminin, or collagen IV, respectively, in the Pial LM. Neurons either detach prematurely or overmigrate through the gaps into the subarachnoid space. A relatively mild cerebellar anomaly is shown in the muscle-eye-brain phenotype shown in **(B)** and **(C)**. Although the vermis is small and dysmorphic, the hemispheres have nearly normal foliation. A few small cysts are present (white arrows in **B** and **C**). The pons contains a midline cleft (black arrow in **C**). The midbrain tectum is large and smooth due to transpial migration of cells. A coronal image through the cerebrum **(D)** shows moderate ventricular enlargement, abnormal hyperintensity of subcortical, and deep white matter, and abnormal sulcation over the convexities; note that the cortex in this region (white arrows) is abnormally thick and seems to be formed of radially oriented bands of neurons. A much more severe Walker–Warburg phenotype is shown in **(E)** and **(F)**. The sagittal image **(E)** shows a thin brain stem with a large kink in the mid pons (black arrowhead), resembling a persistent pontine flexure. The MB tectum is very large and rounded (large black arrow). Only a small vermis (small black arrow) is present. Massive hydrocephalus can be seen, as can a small occipital cephalocele (white arrow). The axial image **(F)** shows an extremely small, dysmorphic cerebellum with no vermis and many cysts within the irregular cortex. Both ocular globes are anomalous.

with disruption of the glial scaffolding and migration of granule cells into the subarachnoid space (Moore et al., 2002). In *Gpr56* deficient mice, glial processes extend through and granule cells migrate through the aforementioned gaps in the glia limitans into the subarachnoid space (Koirala et al., 2009). In human studies, the effects upon the MB-HB vary considerably (Aida et al., 1994; Gelot et al., 1995; van der Knaap et al., 1997; Barkovich, 1998; Clement et al., 2008), with some patients having normal cerebella and others having mild dysgenesis resulting in mild vermian hypoplasia with some alteration of foliation; of note, the severity of cortical dysgenesis is sometimes similar in the cerebrum and cerebellum but the involvement may be discrepant, suggesting that some gene products have different roles in the forebrain and HB. Moderately severe cerebellar dysgenesis consists of a significantly dysmorphic cortex containing cyst-like structures that contain mesenchymal tissue (**Figures 3B–F**) and are connected to the surface via spaces containing penetrating blood vessels (Takada and Nakamura, 1990). This suggests a process similar to that in the cerebrum where cerebral tissues migrates outward and leptomeningeal tissues inward through the defects in the pial limiting membrane **(Figure 3D)**. Most patients with cerebellar dysgenesis have a small pons with a ventral midline cleft (**Figures 3B,C**) (van der Knaap et al., 1997; Barkovich, 1998). In the most severe cases **(Figures 3E,F)**, the cerebellum is extremely small with very dysmorphic cortex and disproportionately small vermis and a small brain stem with "kink" in the mid-pons that is best seen in the sagittal plane (**Figure 3E**); the reason for the small cerebellum might be an absence of dispersion of Purkinje cells (PCs) due to interruption of granule cell migration and consequent absence of Reelin secretion (see next section on cerebellar disorders due to abnormalities of the Reelin pathways). Nearly all affected patients also have abnormalities of the mesencephalic tectum, which is thickened without identifiable collicula due to overmigration of cells (**Figures 3B,E**), and dysmyelination; the reasons for these phenomena are not yet known.

Another malformation complex involving both supra- and infratentorial structures is caused by *mutations of the Reelin pathway*. Reelin is a large glycoprotein that is secreted into the extracellular matrix by Cajal–Retzius cells in the marginal zone of the developing cerebral cortex, where its actions are thought to allow later migrating glutamatergic neurons to pass neurons in deeper cortical layers and to aid in detachment of the neurons from the radial glia at the proper cortical layer (D'Arcangelo et al., 1995; Ogawa et al., 1996; Trommsdorff et al., 1999; Hack et al., 2007; Sentürk et al., 2011). In the hippocampus, Reelin functions in the alignment of pyramidal neurons (Nakajima et al., 1997; Tissir and Goffinet, 2003), and in the cerebellum, Reelin is secreted by the external granular layer and cerebellar nuclear neurons during early development to aid in dispersion of PCs (Miyata et al., 1996; Trommsdorff et al., 1999; Hack et al., 2007; Larouche et al., 2008). In all regions, Reelin action is mediated by binding to specific receptors on target cells, ApoER2, VLDLR, (Miyata et al., 1997; D'Arcangelo et al., 1999; Hiesberger et al., 1999; Trommsdorff et al., 1999; Fink et al., 2006) and ephrin Bs, the transmembrane ligands for Eph receptors (Sentürk et al., 2011). Interaction with Reelin induces VLDLR and ApoER2 to bind the adaptor protein DAB1, which leads to activation of Src family tyrosine kinases (SFKs) and other kinases that phosphorylate DAB1 at its tyrosine residues (Howell et al., 1997; Sheldon et al., 1997; Rice et al., 1998; Bock and Herz, 2003). In the cerebellum, Reelin interacts with receptors on PCs, which respond by dispersing from their clusters in the central cerebellum and migrating to the cerebellar cortex (Trommsdorff et al., 1999; Hack et al., 2007; Sentürk et al., 2011). If the Reelin cascade within the PCs is disrupted, as by mutations of *RELN*, its receptors, or *DAB1*, neither the cerebrum nor the cerebellum form properly, although the precise histological details and mechanisms of the resulting malformations are debated (Bock and Herz, 2003; Larouche et al., 2008; Frotscher, 2010; Boyle et al., 2011; Honda et al., 2011; Sentürk et al., 2011).

Mutations of *RELN* in humans result in congenital lymphedema and hypotonia, impaired cognition, myopia, nystagmus, and generalized epilepsy. Imaging shows a very severe malformation, including thickened cortex, simplified sulcation, hippocampal dysmorphism, and profound cerebellar hypoplasia (**Figures 4A,B**) (Hong et al., 2000). Mutations of *VLDLR* produce a significantly less severe disorder, known as the disequilibrium syndrome (Boycott et al., 2005). In this disorder, children present with delayed motor development and cerebellar ataxia. MRI shows simplified cerebral sulcation (although sulcation is less simplified and cortex less thickened than with *RELN* mutations) and profound cerebellar hypoplasia (**Figure 4C**) (Glass et al., 2005). These patterns of cerebral and cerebellar dysgenesis

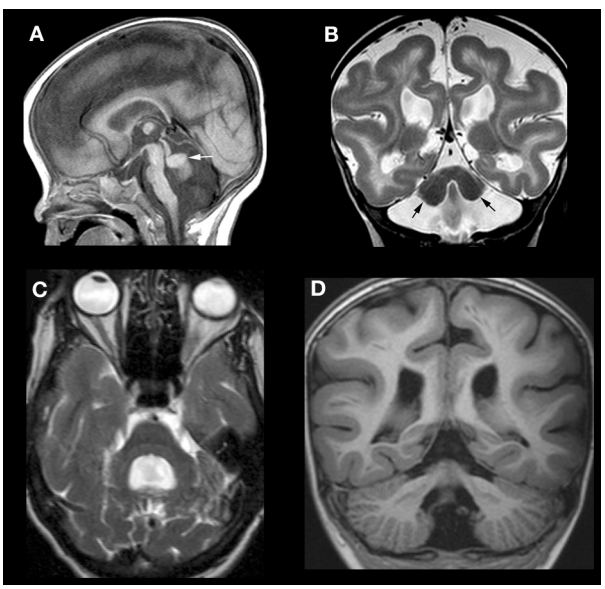

FIGURE 4 | Array of findings in cerebrum and cerebellum of disorders of the Reelin pathway. The most severe situation, with severe RELN depletion results in a very small vermis (white arrow in **A**), small, smooth cerebellar hemispheres (black arrows in **B**), and a thick, pachygyric cerebral cortex **(B)**. *VLDLR* mutation results in a less severe cerebral dysgenesis (cortex is thinner and more sulci are present) but the cerebellum is quite severely affected, both hypoplastic and smooth **(C)**. Severe cerebral but less severe cerebellar involvement (note that the cerebellum is larger and cortex is less smooth) **(D)** can be seen, but the precise mechanism/mutation that results in this appearance is not known.

are consistent with the observed findings in animal models with Vldlr knock-outs [Larouche et al., 2008 #3095];(Trommsdorff et al., 1999; Larouche et al., 2008; Honda et al., 2011; Sentürk et al., 2011). Vldlr mediates a "stop" signal for migrating cerebral cortical neurons; its absence allows overmigration with too many neurons in the molecular layer and mildly abnormal sulcation. Apoer2 is essential for migration of late generated cortical neurons past the earlier generated ones; its absence results in a thicker cortex with less sulcation, more resembling that seen with Reln mutations (Benhayon et al., 2003; Hack et al., 2007). In the cerebellum of Vldlr null animals, a large portion of PCs are not dispersed and remain as heterotopic clusters deep within the hemispheres, whereas Apoer2 null animals have ectopic PCs largely restricted to the anterior vermis, resulting in a much less severe cerebellar dysgenesis (Larouche et al., 2008). No human mutations of ApoER2 have been described in the literature. However, the author has seen MRIs of patients with RELN-like cerebral cortical dysgenesis but mildly abnormal cerebella (**Figure 4D**); testing is underway to determine whether these are caused by mutations of APOER2, DAB1, or some other, as yet unknown, component of the Reelin pathway.

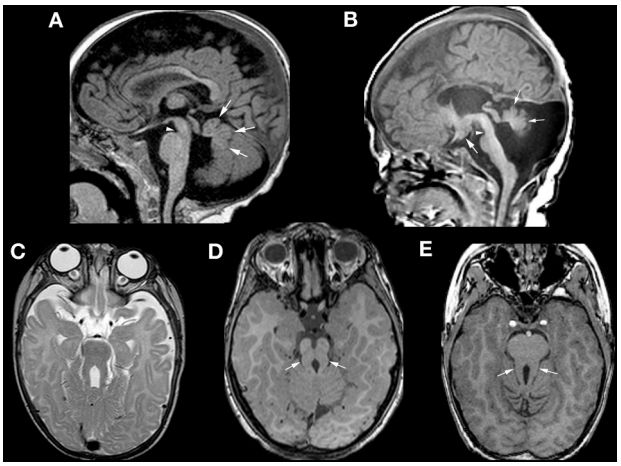

FIGURE 5 | Neuroimaging findings in molar tooth malformations. The characteristic imaging findings of a small vermis (small white arrows, **A,B**) and narrow isthmus (small white arrowhead, **A,B**) are identified on sagittal images. A tuber cinereum hamartoma is seen in **(B)**. The variable appearances of the "molar tooth," resulting from the large, horizontal superior cerebellar peduncles, are shown (white arrows in **C, D,** and **E**).

DISORDERS OF CEREBELLAR DYSGENESIS

Another well-defined syndrome of cerebellar dysgenesis is the Joubert Syndrome and its Related Disorders (JSRD, often called Molar Tooth Malformations). A familial syndrome of "agenesis of the cerebellar vermis" was first described by Joubert et al. in 1969; affected patients had episodic hyperpnea in infancy, abnormal eye movements, ataxia, and cognitive impairment (Joubert et al., 1969). The disorder was further elucidated in several papers by Boltshauser and his colleagues (Boltshauser and Isler, 1977; Boltshauser et al., 1981; Steinlin et al., 1997), who described a variable degree of vermian hypoplasia (rather than agenesis) and multiple other features, with variable outcomes, in affected patients. The advent of MRI revealed a characteristic "molar tooth" appearance of the MB (**Figure 5**) (Maria et al., 1997; Quisling et al., 1999). This appearance was soon found in many disorders with widely varying phenotypic features of other organs including the eyes, kidneys, liver, and extremities (Egger et al., 1982; Houdou et al., 1986; Chance et al., 1999; Satran et al., 1999; Haug et al., 2000; Gleeson et al., 2004), suggesting that these heretofore seemingly distinct disorders were in some way related. These disorders were very curious, as no common thread could be found among the processes involved, even within the same organs. Within the nervous system, it was difficult to find the common underlying cause connecting the disorders of retina development, vermian hypoplasia, aberrant white matter pathways (neither the corticospinal tracts nor the superior cerebellar peduncles decussate properly), occasional polymicrogyria and hypothalamic harmartomas (**Figure 5**) (Haug et al., 2000; Zaki et al., 2008; Giordano et al., 2009; Harting et al., 2011). It was also noteworthy that the characteristic molar tooth sign had many different appearances with the molar "roots" (composed of the superior cerebellar peduncles) sometimes thick, sometimes thin, sometimes straight and sometimes curved; clearly a lot of different processes were going on. Answers began to emerge when it was discovered that all of the genes implicated in these disorders are associated with the function of the primary cilium/basal body organelle, a structure that is present in many cell types, including renal tubule epithelial cells, retinal photoreceptors, chondrocytes, fibroblasts, and neurons (Arts et al., 2007; Chizhikov et al., 2007; Delous et al., 2007; Frank et al., 2008; Gorden et al., 2008; Spassky et al., 2008; Doherty, 2009). Ciliary membranes contain receptors and ion channel proteins mediating cell signaling, including roles for SHH, WNT, and PDGFa signaling pathways that control diverse processes (e.g., cell differentiation, migration, axonal pathfinding, and planar cell polarity). SHH binding to its transmembrane receptor PTCH abolishes the inhibitory effect of PTCH on SMO, resulting in localization of SMO to the primary cilium, and transduction of signals to the nucleus through the GLI transcription factors. The result is de-repression and activation of SHH target genes (Satir and Christensen, 2007). The SHH pathway is important for dorsal-ventral patterning of the neural tube and, later, for proliferation of cerebellar granule cells (Wechsler-Reya and Scott, 1999; Huangfu et al., 2003). However, reduction of granule cell proliferation cannot in itself explain the vermian hypoplasia seen in the MTMs (Chizhikov et al., 2007; Spassky et al., 2008), as the effect of SHH is diffuse, involving both vermis and hemispheres, but the vermis is very disproportionately involved in MTMs. A more likely explanation is decreased Wnt reporter activity, accompanied by reduced proliferation, at the site of hemispheric fusion, as was reported in the developing cerebellum of Ahi1-mutant mice (Lancaster et al., 2011); this phenotype was partially rescued by treatment with lithium, a Wnt pathway agonist (Lancaster et al., 2011). The wide phenotypic variation among families harboring mutations in genes encoding ciliary proteins also suggests that genetic modifiers are important in determining specific features within the ciliopathy spectrum (Davis et al., 2011; Zaki et al., 2011). This interesting group of disorders has much more to teach us about both development and disorders thereof.

Many other disorders of the MB-HB have been described in association with supratentorial anomies, including cerebellar hypoplasia associated with severe variants of cerebral lissencephaly secondary to alpha-A1 tubulin (TUBA1A) and other tubulin mutations (Poirier et al., 2007; Kumar et al., 2010), cerebellar hypoplasia associated with postmigrational microcephaly secondary to mutations of calcium modulated-dependent serine protein kinase (CASK), which is associated with X-linked mental retardation (Najm et al., 2008) and cerebellar hypoplasia associated with midline brain stem clefts and agenesis of the corpus callosum, presumably resulting from mutations preventing midline axonal crossing (Barkovich et al., 2009). Application of discoveries from developmental neuroscience will aid our understanding of these disorders, and what is learned from studying these disorders will help us to better understand brain development.

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The genesis of cerebellar GABAergic neurons: fate potential and specification mechanisms

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All cerebellar neurons derive from progenitors that proliferate in two germinal neuroepithelia: the ventricular zone (VZ) generates GABAergic neurons, whereas the rhombic lip is the origin of glutamatergic types. Among VZ-derivatives, GABAergic projection neurons, and interneurons are generated according to distinct strategies. Projection neurons (Purkinje cells and nucleo-olivary neurons) are produced at the onset of cerebellar neurogenesis by discrete progenitor pools located in distinct VZ microdomains. These cells are specified within the VZ and acquire mature phenotypes according to cell-autonomous developmental programs. On the other hand, the different categories of inhibitory interneurons derive from a single population of Pax-2-positive precursors that delaminate into the prospective white matter (PWM), where they continue to divide up to postnatal development. Heterotopic/heterochronic transplantation experiments indicate that interneuron progenitors maintain full developmental potentialities up to the end of cerebellar development and acquire mature phenotypes under the influence of environmental cues present in the PWM. Furthermore, the final fate choice occurs in postmitotic cells, rather than dividing progenitors. Extracerebellar cells grafted to the prospective cerebellar white matter are not responsive to local neurogenic cues and fail to adopt clear cerebellar identities. Conversely, cerebellar cells grafted to extracerebellar regions retain typical phenotypes of cerebellar GABAergic interneurons, but acquire type-specific traits under the influence of local cues. These findings indicate that interneuron progenitors are multipotent and sensitive to spatiotemporally patterned environmental signals that regulate the genesis of different categories of interneurons, in precise quantities and at defined times and places.

Keywords: GABAergic interneuron, Pax-2, fate specification, heterotopic–heterochronic transplantation, Purkinje cell, ventricular zone, prospective white matter, Ascl-1

INTRODUCTION

The great attention paid in the last decades to the study of the basic mechanisms of neuropsychiatric disorders, such as autism, schizophrenia, epilepsy, and mental retardation, has contributed to highlight the pathogenic role played, together with other concurrent factors, by the unbalanced ratio between excitatory and inhibitory transmission in different CNS regions (Levitt et al., 2004; Di Cristo, 2007; Sun, 2007). GABAergic elements are most important to ensure an integrative and synchronized support for information processing in the brain (Möhler et al., 2004; Bartos et al., 2007). GABAergic signaling also regulates essential ontogenetic phases, from neuronal proliferation, migration, and differentiation (Owens and Kriegstein, 2002), to experience-dependent refinement of local circuits (Hensch, 2005). Inhibitory interneurons play a fundamental regulatory role on cortical development and plasticity, as they contribute to the onset, closure, and outcome of critical periods (Hensch, 2004). Therefore, understanding the developmental processes that regulate the generation of inhibitory interneurons and their assembly into neural networks is crucial to elucidate a broad range of physiologic and pathologic conditions.

Despite the wide variety of inhibitory interneurons present in the different subdivisions of the CNS, one major strategy applies to ensure the production of appropriate quantities of distinct interneuron subtypes. In most CNS regions, different categories of interneurons are generated by discrete pools of fate-restricted progenitors that become specified within defined germinal sites at precise developmental stages (Caspary and Anderson, 2003; Helms and Johnson, 2003; Bovetti et al., 2007; Batista-Brito et al., 2008; Wonders et al., 2008; Batista-Brito and Fishell, 2009). Recent findings, however, indicate that the variety of cerebellar GABAergic interneurons originates from a single population of multipotent progenitors that persist throughout embryonic and postnatal development and adopt different mature identities in response to local instructive cues. In the next sections we will first describe the general features of cerebellar neurogenesis and, then, we will focus on the mechanisms governing the generation of GABAergic projection neurons and interneurons.

ORIGIN OF CEREBELLAR PHENOTYPES

In the mouse, cerebellar histogenesis starts at embryonic day 9 (E9), after the establishment of regional specification and the formation of two germinal neuroepithelia that line the opening of the fourth ventricle: the rhombic lip (RL), located at the roof plate, and the ventricular zone (VZ), located at the inner germinal

layer (Hatten and Heintz, 1995). The two neuroepithelia disappear at birth, but dividing progenitors emigrate from them and give rise to secondary germinal sites, where neurogenesis continues up to postnatal development. Hence, RL progenitors move along the cerebellar surface to form the external granular layer (EGL), whereas those of the VZ delaminate into the prospective white matter (PWM), which surrounds the deep nuclei and extends into the axis of the nascent folia.

According to the classical descriptions of cerebellar neurogenesis, the VZ was the source of deep cerebellar nuclei (DCN) neurons, Purkinje cells (PCs), and Golgi cells, whereas the EGL was the origin of granule neurons and inhibitory interneurons of the molecular layer (ML; Ramón Y Cajal, 1911; Altman, 1972). Later, it was unequivocally demonstrated that EGL progenitors exclusively generate granule cells (Hallonet et al., 1990; Hallonet and Le Douarin, 1993; Gao and Hatten, 1994). Furthermore, experiments using retroviral vectors (Zhang and Goldman, 1996a,b) together with clonal analyses (Mathis et al., 1997; Milosevic and Goldman, 2002, 2004; Mathis and Nicolas, 2003) showed that inhibitory interneurons derive from progenitors that proliferate in the PWM during late embryonic life and postnatal development. Finally, recent observations disclosed a precise neurochemical compartmentalization of cerebellar progenitors in the two primary germinal sites. RL precursors, which express the mouse homolog of *Drosophila* Atonal, Math-1, generate all glutamatergic types, including DCN projection neurons, unipolar brush cells, and granule cells, whereas VZ progenitors, distinguished by the expression of the pancreas transcription factor 1-a (Ptf1-a) produce all the GABAergic phenotypes, including PCs, nucleo-olivary projection neurons, and all the inhibitory interneurons (Hoshino et al., 2005; Machold and Fishell, 2005; Wang et al., 2005; Englund et al., 2006; Fink et al., 2006).

NEUROGENESIS IN THE RL

Two different regions can be distinguished in the RL: a rostral part that specifically gives rise to cerebellar glutamatergic neurons and a caudal one that generates the projection neurons of the major precerebellar nuclei (Alcántara et al., 2000; Wingate, 2001; Wang et al., 2005). A series of genetic fate mapping studies have finely described the temporal sequence of appearance of Math-1-positive progenitors fated to different cerebellar districts (Wingate and Hatten, 1999; Lin et al., 2001; Wingate, 2001; Machold and Fishell, 2005; Machold et al., 2007). Math-1 expression begins in the murine RL at E9.5 (Akazawa et al., 1995) and is dynamically regulated by the antagonistic interaction between Notch1 signaling in the cerebellar primordium and the bone morphogenetic proteins secreted by the roof plate (Machold et al., 2007). Math-1 provides fundamental instructive information for the generation of cerebellar RL precursors. Mice carrying a Math-1 targeted deletion lack the rostral RL derivatives, whereas neurogenesis of caudal precursors is unaffected (Akazawa et al., 1995; Ben-Arie et al., 1997; Fünfschilling and Reichardt, 2002). Three main waves of fate-restricted cerebellar precursors sequentially leave the RL. The first one, from E10.5 to E12.5, gives rise to glutamatergic projection DCN neurons that migrate rostrally toward a subpial position, in the so called "nuclear transitory zone" (Machold and Fishell, 2005; Wang et al., 2005; Fink et al., 2006; Machold et al., 2007). Secondly, progenitors of unipolar brush cells, glutamatergic interneurons of the granular layer (GL), leave the germinal site between E14 and E21 (Nunzi et al., 2001, 2002; Sekerkovà et al., 2004) through two distinct streams of dorsally and rostrally migrating cells (Englund et al., 2006). Finally, a third migratory wave from the RL is composed by granule cell progenitors, which spread tangentially along the cerebellar surface to form the EGL in late embryogenesis and early postnatal life (Machold and Fishell, 2005). It has been recently shown that some granule cells may also derive from GFAP-expressing progenitors, which reside in the EGL during the first two postnatal weeks (Silbereis et al., 2010).

NEUROGENESIS IN THE VZ

Ventricular zone cells give origin to all cerebellar GABAergic neurons according to a two-step sequence: projection neurons (nucleo-olivary neurons and PCs) are generated at the onset of cerebellar neurogenesis by precursor cells that become specified within the germinal layer. Interneurons derive from VZ progenitors that delaminate into the PWM, where they continue to divide and take their final phenotypic choices (**Figure 1**; Zhang and Goldman, 1996a,b; Maricich and Herrup, 1999). Accordingly, the VZ comprises several cell subpopulations, distinguished by specific expression profiles of transcription factors and proneural genes that induce GABAergic specification or direct fate choices toward projection neurons or interneurons.

All VZ progenitors express the transcription factor Ptf1-a, which is required for the initial specification of the GABAergic

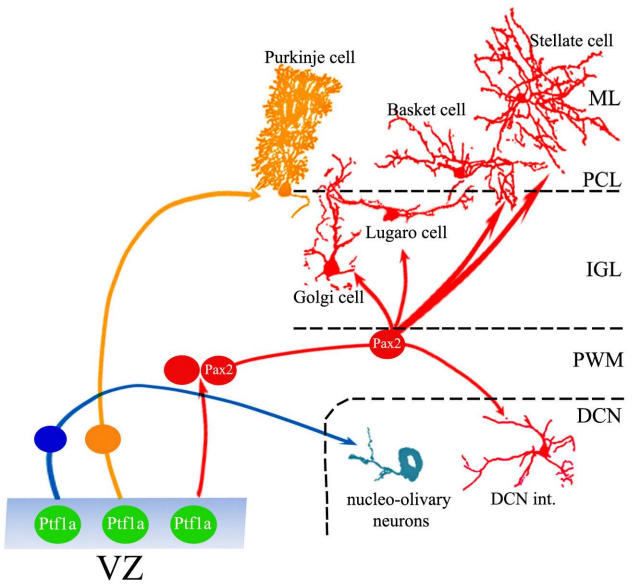

FIGURE 1 | Generation of cerebellar GABAergic neurons. Ptf1-a-positive progenitors in the VZ generate the full repertoire of GABAergic projection neurons and interneurons through different neurogenic strategies. While projection neurons are generated from precursors that proliferate and become specified within the VZ, interneurons derive from a population of dividing cells that originate in the VZ, but continue their neurogenic activity in the PWM, where they acquire mature identities under the influence of local cues. ML, molecular layer; PCL, Purkinje cell layer; IGL, internal granular layer; PWM, prospective white matter; DCN, deep cerebellar nuclei.

lineage (Hoshino et al., 2005; Hoshino, 2006). In Ptf1-a knockout mice, some VZ progenitors migrate to the EGL and acquire granule cell phenotypes, indicating that Ptf1-a is needed to prevent VZ progenitors from engaging into a default granule cell developmental program (Pascual et al., 2007). The recent observation that Ptf1-a is mainly expressed in postmitotic VZ cells (Huang et al., 2010) suggests that this factor may act by inducing progenitors to leave the cycle and engage into GABAergic differentiation. This mechanism, however, cannot be immediately reconciled with the genesis of interneurons, which also derive from Ptf1-a-positive VZ cells that continue to proliferate in the PWM, where Ptf1-a is down-regulated.

While it is clear that Ptf1-a expression is directly involved in the choice between GABAergic or glutamatergic fates, the lineage relationships linking different VZ-derivatives are only partially known. For instance, although fate mapping analyses suggest that PCs, DCN neurons, and interneurons may be clonally related (Mathis et al., 1997), there is still no direct evidence that all GABAergic types actually derive from a single pool of VZ progenitors. It is now firmly established that the VZ comprises a mosaic of spatially discrete microdomains, distinguished by specific gene expression profiles, which are thought to be the origin of different populations or subpopulations of GABAergic neurons (Chizhikov et al., 2006; Morales and Hatten, 2006; Salsano et al., 2007; Sillitoe and Joyner, 2007; Zordan et al., 2008; Lundell et al., 2009; Mizuhara et al., 2009; Dalgard et al., 2011; Sudarov et al., 2011). Such VZ subdivisions are evident from the outset of cerebellar neurogenesis and persist throughout embryonic life, but undergo a characteristic evolution of their spatial arrangement (e.g., Zordan et al., 2008). Therefore, while the presence of microdomains is a landmark of VZ organization, it is still unclear whether VZ cells are a mosaic of distinct subpopulations that maintain steady properties throughout embryonic life, or represent a single pool of functionally dynamic cells, which modify their transcription profiles and acquire novel developmental potentialities at subsequent ontogenetic stages.

Ventricular zone cells are characterized by the expression of Neurogenin-1 (Ngn-1), Neurogenin-2 (Ngn-2), and Ascl-1 (Zordan et al., 2008). Ngn-1 and Ngn-2 are transiently expressed between E10.5 and E13.5 in distinct microdomains. In contrast, Ascl-1 is expressed during a longer time-window throughout the entire germinal layer (Zordan et al., 2008). Genetic fate mapping (Lundell et al., 2009; Kim et al., 2011) and examination of knockout animals (Dalgard et al., 2011) indicate that Ngn-1-positive progenitors are primarily fated to become projection neurons (notably PCs from E10.5 to E12.5), but they also contribute to generate interneurons at later times. Similarly, Ngn-2, which is a direct downstream target of the transcription factor complex Ptf1-J in the spinal cord (Henke et al., 2009), also appears to be mainly involved in the generation of projection neurons (Florio et al., 2011). Finally, genetic fate mapping analysis with Ascl-1CreER^{T2} transgenic mice indicates that this gene is transiently expressed by all VZ-derived neuronal and glial phenotypes, with different roles in their generation (Sudarov et al., 2011). Expression of Ascl-1 also occurs in PWM cells, including those derived from the VZ (Kim et al., 2008; Grimaldi et al., 2009; Sudarov et al., 2011), indicating that this factor also participates to neurogenic phenomena that occur at later ontogenetic stages (see below).

Among the VZ microdomains, some are related to the generation of different classes of PCs, whereas others define germinal regions destined to produce interneurons (Zordan et al., 2008; Lundell et al., 2009). To date, the only selective and specific marker for all maturing cerebellar GABAergic interneurons is Pax-2 (Maricich and Herrup, 1999). The first Pax-2-positive cells appear at E12.5 in a restricted region at the medial aspect of the VZ (Maricich and Herrup, 1999; Zordan et al., 2008). At this stage, the Pax-2-positive spatial domain is mutually exclusive with that of Ngn-1 and Ngn-2, while it is covered by Ascl-1 (Zordan et al., 2008). VZ progenitors also bear the cell surface marker Neph3, a downstream target of Ptf1-a (Mizuhara et al., 2009). Two subsets of Neph3-positive cells can be distinguished on the basis of high or low E-cadherin expression levels, which correspond to the progenitors of PCs and GABAergic interneurons, respectively (Mizuhara et al., 2009). Finally, another feature that can be used to discriminate between the two lineages is the differential sensitivity of VZ progenitors to sonic hedgehog (Shh): Shh signaling stimulates the proliferation of Blbp-positive radial glia (Anthony et al., 2005) to expand the pool of GABAergic interneuron progenitors, whereas it does not affect the generation of PCs (Huang et al., 2010).

NEUROGENESIS AND GLIOGENESIS IN THE PWM

The cerebellar PWM is a secondary germinal site, active during late embryonic life and postnatal development. The cellular composition of the PWM is complex, including cells with stem-like properties (Klein et al., 2005; Lee et al., 2005), dividing progenitors, and distinct categories of postmitotic elements at different maturation stages (**Figure 2**). Proliferating cells comprise progenitors that delaminated from the VZ or immigrated from extracerebellar sources. On the whole, PWM cells give rise to GABAergic interneurons, astrocytes, and oligodendrocytes (**Figure 2**; Zhang and Goldman, 1996b; Milosevic and Goldman, 2002, 2004; Grimaldi et al., 2009). Also in this case, however, the relationships linking these lineages are largely unclear.

In the PWM, Ascl-1 is active in two distinct cell populations: Olig2-positive cells show strong expression of Ascl-1, whereas Pax-2-positive cells show weaker expression (Grimaldi et al., 2009). Consistent with the idea that this gene is involved in the genesis of interneurons and oligodendrocytes, both Ascl-1 null mice (Grimaldi et al., 2009) and conditional knockout mice (Sudarov et al., 2011) show a dramatic decrease of these cell types accompanied by increased numbers of astrocytes. Transplantation experiments (Grimaldi et al., 2009), corroborated by recent analysis of chick–quail chimeras (Mecklenburg et al., 2011), show that the majority of cerebellar oligodendrocytes actually derive from extracerebellar sources. Therefore, the loss of interneurons and the concomitant gain of astrocytes seen in Ascl-1 mutants suggest that these two lineages may be related, and Ascl-1 is involved in regulating their genesis. Overexpression of Ascl-1 at E15 yields high numbers of Pax-2-positive cells at the expense of astrocytes, suggesting that these two cell types derive from a common precursor whose fate choice toward the interneuron phenotype is directed by Ascl-1 (Grimaldi et al., 2009). In line with this view, fate mapping analysis of GFAPCreER^{T2} mice shows that cerebellar GABAergic

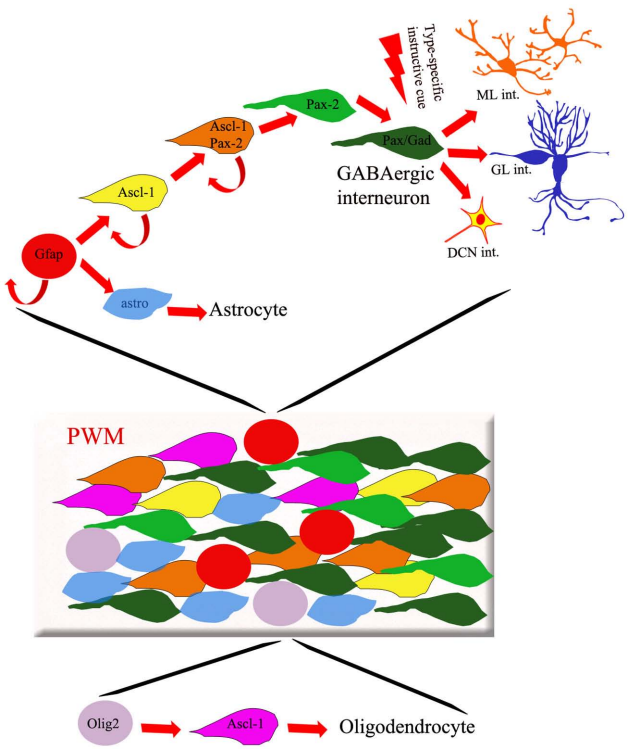

FIGURE 2 | Cellular composition of the PWM. The PWM contains different types of dividing progenitors and postmitotic cells fated to originate GABAergic interneurons, astrocytes, and oligodendrocytes. Whereas the latter cells derive from Olig-2-expressing progenitors (purple circles), GFAP-positive precursors (red circles) are thought to generate both inhibitory interneurons and astrocytes. The production of different subtypes of GABAergic interneurons is regulated by spatio-temporally patterned environmental influences, present in the PWM environment and acting on postmitotic Pax-2/Gad67-positive cells.

interneurons derive from a GFAP-positive precursor that could be shared with astrocytes (**Figure 2**, Silbereis et al., 2009). In spite of these observations, however, the unequivocal identification of a common progenitor for interneurons and astrocytes is still missing.

Among PWM cells, Pax-2 is the distinctive marker of the interneuron lineage. The original report indicated that Pax-2 is expressed by actively dividing interneuron progenitors (Maricich and Herrup, 1999). More recently, however, it has been shown that Pax-2 is upregulated when PWM progenitors enter their last cell division (Weisheit et al., 2006; Leto et al., 2009), implying that the generation of appropriate numbers of interneurons must be sustained by the proliferation of Pax-2-negative elements (Weisheit et al., 2006; Schilling et al., 2008). A recent study suggests that RBP-J signaling converts a population of Sox-2-positive/Pax-2-negative precursors into Pax-2-positive interneuron progenitors (Komine et al., 2011). Other environmental factors, such as basic fibroblast growth factor (Lee et al., 2005), Shh (Huang et al., 2010), or thyroid hormones (Manzano et al., 2007), also contribute to regulate the production rates of interneurons. Similarly, cell-intrinsic mechanisms that control cell cycle progression and length also appear to be relevant for the proper outcome of these processes (Lee et al., 2009; Leto et al., 2011).

Following their last mitosis, Pax-2-positive cells remain in the PWM for a rather long time (up to several days), before moving to their final location in the DCN or cortex (Leto et al., 2009). During this period, the young interneurons progress in their maturation. For instance, expression of the GABA synthesizing enzyme glutamic acid decarboxylase only occurs about 24 h after the completion of the last cycle. The reason for such a long "waiting period" of young interneurons in the PWM is not clear. It must be noted that if Pax-2-expressing cells are positively committed to the GABAergic fate, young postmitotic interneurons are still able to switch their fate and adopt host-specific interneuron identities following heterotopic/heterochronic transplantation (see below; Leto et al., 2009). These considerations suggest that while Pax-2 upregulation during the last mitosis marks the commitment of the cell toward the GABAergic interneuron destiny, the final fate choice toward distinct mature phenotypes is taken during the ensuing period, when the young interneurons sojourn in the PWM (**Figure 2**).

SPATIO-TEMPORAL EVOLUTION OF THE DEVELOPMENTAL POTENTIAL OF CEREBELLAR PROGENITORS

One of the most convenient ways to elucidate the relative contribution of cell intrinsic and environmental factors in the specification of neuronal phenotypes is to examine the fate of cells exposed to heterotopic and/or heterochronic environmental conditions. This approach is particularly suitable in the case of the genesis of cerebellar GABAergic neurons, given the peculiar time-sequence of phenotypic generation and the existence of two distinct germinal sites, the VZ and the PWM.

In this context, a first major question is whether postnatal progenitors, normally destined to generate interneurons, can switch their fates and become projection neurons when exposed to the environment of the embryonic cerebellar primordium. In a first study devoted to address this issue, morsels of postnatal (P4) and embryonic (E12) mouse cerebella were mixed together and co-grafted to the cerebellum or to the forth ventricle of adult hosts (Jankovski et al., 1996). To identify donor cells derived from either sources, postnatal tissues were isolated from transgenic mice expressing the LacZ reporter gene in different neuron populations. In such mixed grafts, the embryonic donor tissue developed typical minicerebellar structures (Sotelo and Alvarado-Mallart, 1991; Rossi et al., 1992), while postnatal cells were incorporated as individual elements within this framework. The latter cells exclusively differentiated into ML interneurons and granule cells (the P4 tissue also contained the EGL), indicating that they were strictly committed toward late-generated identities and not responsive to neurogenic cues provided by the embryonic milieu (Jankovski et al., 1996).

In a later study, to gain further insights on the spatio-temporal evolution of the fate potentiality of cerebellar progenitors, E12 or P4 donors tagged by green fluorescent protein (GFP) expression were dissociated to single cell suspensions and injected into the cerebral ventricles of E15 rat embryos or into E12 organotypic explants (Carletti et al., 2002). Donor cells of both ages engrafted in the cerebellum and in many extracerebellar sites of

the recipient brain. Even in ectopic locations, however, these cells exclusively differentiated in cerebellar phenotypes, showing that cerebellar cells are strictly regionally specified already at E12. The phenotypic repertoires yielded by grafted cells differed according to their age: E12 donors developed the full repertoire of cerebellar phenotypes, including both projection neurons and interneurons, whereas postnatal cells only produced granule cells and GABAergic interneurons.

These experiments show that postnatal cerebellar progenitors are unable to adopt the identities of projection neurons, even when exposed to the embryonic cerebellar milieu. This suggests that the sequence of phenotypic generation during cerebellar neurogenesis is achieved by progressively reducing the developmental potentialities of progenitors. This outcome could be obtained either by restricting the differentiation properties of a single population of initially multipotent precursors, or by creating *ab initio* distinct subsets of fate-restricted progenitors destined to generate different mature types at subsequent developmental stages. The early compartmentalization of the germinal neuroepithelia as well as direct experimental evidence indicates that the latter case applies to certain types of cerebellar neurons, and notably RL derivatives (Gao and Hatten, 1994; Alder et al., 1996). On the other hand, the clonal relationship that may link PCs and inhibitory interneurons (Mathis et al., 1997; Mathis and Nicolas, 2003) suggests that VZ progenitors are initially competent to generate the whole phenotypic repertoire, but their potential becomes restricted at more advanced developmental stages, when VZ cells emigrate to the PWM.

ONE FOR ALL: MULTIPLE TYPES OF GABAergic INTERNEURONS FROM A SINGLE POOL OF PROGENITORS

Inhibitory interneurons of the cerebellar cortex and DCN can be subdivided in several classes on the basis of their morphological, neurochemical, and functional properties (Schilling et al., 2008; Schilling, 2011). While basket and stellate cells in the ML are thought to be extreme variants of the same category (Rakic, 1972; Sultan and Bower, 1998; Koscheck et al., 2003), several subtypes of Golgi neurons populate the GL together with Lugaro, globular, and candelabrum cells (Geurtz et al., 2001; Lainé and Axelrad, 2002; Simat et al., 2007). DCN interneurons may also comprise different subtypes (Uussisari and Knöpfel, 2011), but these are still incompletely characterized (Schilling, 2011).

The entire repertoire of inhibitory interneurons derive from Pax-2-expressing cells that are continuously generated between E12.5 and P15, with a peak around P5, leading to the production of 75% of all the interneurons prior to P7 in the mouse (Weisheit et al., 2006; Leto et al., 2008). In the rodent cerebella the genesis of GABAergic interneurons is completed by the end of the second postnatal week, but in the rabbit Pax-2-positive cells continue to be generated for a considerably longer time by progenitors lying in the subpial layer (Ponti et al., 2008, 2010).

In all instances, GABAergic interneurons are produced during a considerably long period from a population of Pax-2-expressing cells. Different mechanisms could be envisaged to explain the unfolding of this process: (1) Pax-2-positive cells might comprise distinct subsets of fate-restricted progenitors committed to different interneuron phenotypes; (2) a single set of initially multipotent progenitors could be progressively restricted in their potentialities, so to generate distinct classes of interneurons in sequence; (3) a single population of multipotent progenitors may retain full potentialities throughout development and make phenotypic choices in response to extrinsic instructive information.

As already mentioned, analyses of clonal composition during cerebellar development suggest that Golgi, basket, and stellate cells may descend from a common lineage (Mathis et al., 1997; Mathis and Nicolas, 2003). These findings, however, do not rule out the existence of spatially segregated pools of fate-restricted cells or the progressive loss of developmental potentialities. To elucidate whether progenitors located in different regions of the postnatal cerebellum (e.g., the central white matter or the axial core of cortical lobules) have distinct developmental potentialities, cells isolated from the periventricular or the subcortical PWM of P1 cerebella were transplanted either to E15 embryos *in utero* or P7 pups *in vivo*. Donor cells from both origins yielded the same phenotypic repertoires, whose composition consistently depended on the age of the recipient cerebellum: the entire variety of interneurons was generated in embryonic recipients, whereas only late-born ML interneurons were produced in postnatal cerebella (Leto et al., 2006). These results show that all interneuron progenitors have the same potentialities regardless of their position. To ask whether the fate potential of these progenitors is progressively restricted as development advances, the types of interneurons produced by E14 or P7 donor progenitors were compared following heterochronic transplantation to recipient cerebella of different embryonic or postnatal ages. Also in these experiments, grafted cells, regardless of their donor age, produced the same phenotypic repertoires, typical of the recipient age (Leto et al., 2006). Together, these experiments indicate that all interneuron progenitors share similar developmental potentialities, which are not dependent on their spatial location or ontogenetic stage. In addition, the consistent acquisition of host-specific identities in both heterotopic and heterochronic conditions indicates that such progenitors are multipotent and make their fate choices under the influence of environmental cues.

SPECIFICATION OF GABAergic INTERNEURONS IN THE PWM

The different categories of interneurons are generated during largely overlapping time windows, according to a precise inside-out sequence, starting with cells destined to the DCN and progressing to those of the GL and ML (Miale and Sidman, 1961; Altman and Bayer, 1997; Yamanaka et al., 2004; Weisheit et al., 2006; Schilling et al., 2008). A salient feature of this process is the strict correlation between interneuron birthdates and placements: in the cortical layers earliest-born interneurons are located deeper, whereas later-born elements settle in progressively more superficial positions (Leto et al., 2009).

Heterochronically transplanted cerebellar cells become mature interneurons that match the phenotype and position of their endogenous counterparts generated at the time of transplantation, suggesting that the donor cells entrain into the recipient neurogenic mechanism and become specified soon after graft (Leto et al., 2009). In line with this conclusion, at short term after transplantation donor cells are found in the PWM and acquire mature traits according to the same times and modes of local interneurons (Leto

et al., 2009). This behavior is not followed by other types of cerebellar cells, such as Purkinje or granule neurons, that settle into the recipient tissue through unusual routes and differentiate according to cell-intrinsic mechanisms (Sotelo and Alvarado-Mallart, 1991; Grimaldi et al., 2005; Carletti et al., 2008; Williams et al., 2008).

Interestingly, also the temporal pattern of Ascl-1 expression in interneuron progenitors correlates with their final placement in the cerebellar cortex (Sudarov et al., 2011). Similar to Pax-2, Ascl-1 is expressed by interneuron progenitors close to their final cycle (Sudarov et al., 2011). On the whole, these findings suggest that phenotypic specification and laminar fate of interneuron progenitors are determined at the time of their last mitosis. Quite surprisingly, however, this conclusion is contradicted by the behavior of juvenile interneurons grafted to heterochronic hosts (Leto et al., 2009). Postmitotic interneurons, identified by BrdU incorporation before cell dissection or by FACsorting of GAD67–GFP-tagged cells (Yamanaka et al., 2004), are still able to switch their fate and adopt identities typical of the recipient age (Leto et al., 2009). As a consequence, although phenotype and position are temporally related to the cell birthdate, specification to distinct mature types actually occurs after the completion of the last cell division.

Even if the final fate choice occurs in postmitotic cells, mechanisms that operate during the last cycle may influence the developmental potential of interneuron precursors. For instance, inactivation of Xrcc1, a DNA repair protein, produces a dramatic loss of GABAergic interneurons due to p53-dependent cell cycle arrest occurring at the moment when progenitor cells start to differentiate (Lee et al., 2009). Likewise, mice lacking the G1-phase active protein cyclin D2 show a severe loss of stellate cells (Huard et al., 1999) and a severe delay in interneuron maturation (Leto et al., 2011). At least for the latter mice, however, the progenitors' capacity to acquire distinct mature phenotypes is not affected (Leto et al., 2011). Therefore, precise regulation of cell cycle dynamics appears to be more relevant in determining the rate of interneuron production rather than controlling phenotype specification.

The neurogenic properties of the PWM milieu have been investigated by transplantation of postnatal cerebellar cells to adult hosts, after the end of interneuron genesis. In these experiments, dissociated donor cells exclusively generated stellate neurons positioned in the outermost regions of the ML. On the contrary, solid pieces of PWM implanted in the recipient parenchyma yielded high quantities of interneurons scattered throughout the white matter and cortical layers. Most interestingly, these solid PWM grafts exposed to the non-neurogenic environment of the adult cerebellum, yielded interneuron repertoires consistent with the donor age: P1 tissue produced both GL and ML interneurons, whereas P7 tissue exclusively generated the latter types (Leto et al., 2009). Thus, in the absence of local neurogenic information in the adult host cerebellum (Grimaldi and Rossi, 2006), the donor PWM appears to be competent to generate different types interneurons in a stage-specific manner.

The nature of the instructive signals contained in the PWM milieu remains elusive. Gene expression analysis of interneuron progenitors failed to reveal distinct subtype-specific expression patterns, further confirming the view that the variety of GABAergic interneurons is mainly determined by local signaling (Glassmann et al., 2009). The latter likely includes the concurrent contribution of cell–cell interactions, diffusible cues, and position-dependent information provided by components of the extracellular matrix (Leto et al., 2008, 2010; Schilling et al., 2008; Schilling, 2011). In any case, the molecular mediators of these processes and their mechanisms of action remain to be established.

NEUROGENIC PROPERTIES OF THE PWM AND ADAPTIVE CAPABILITIES OF INTERNEURON PROGENITORS

The findings discussed in the previous sections indicate that the generation of different types of interneurons depends on the sensitivity of the progenitors to instructive cues present in their surrounding microenvironment. Another suitable way to investigate the neurogenic properties of the developing cerebellar milieu is the transplantation of foreign progenitors. Following *in utero* injection, E12 neocortical cells engraft in many different CNS regions, including the cerebellum (Carletti et al., 2004). However, while these donors acquire site-specific identities in different telencephalic sites, in the cerebellum they fail to develop any cerebellar-specific traits and show poor integration capabilities.

More recently, it was reported that progenitors from the forebrain subventricular zone transplanted to the postnatal PWM acquire anatomical and neurochemical features of cerebellar interneurons (Milosevic et al., 2008). Later on, however, comparison of the fates of progenitors isolated from different sites along the neuraxis (lateral ganglionic eminence, telencephalic subventricular zone, ventral mesencephalon, dorsal spinal cord) led to partially different conclusions (Rolando et al., 2010). In the latter transplants, extracerebellar progenitors did not express cerebellar-specific regulatory genes and failed to acquire clear cerebellar phenotypes and positions consistent with the recipient age. Interestingly, some of the grafted neurons developed peculiar morphological features, such as particular arrangements of their dendrites, as a result of their interaction with the surrounding architecture of host cerebellum. Hence, while extracerebellar cells are not sensitive to neurogenic signals provided by the PWM, they are able to adapt to local constraints and acquire some mature traits that are typical of the recipient tissue.

Heterotopic transplantation to extracerebellar sites is also a useful tool to investigate the intrinsic developmental properties of interneuron progenitors. As remarked above, cerebellar cells implanted *in utero* settle in wide areas of the recipient CNS, but exclusively acquire cerebellar phenotypes (Carletti et al., 2002). Namely, both embryonic and postnatal donors produce different types of GABAergic interneurons that can be readily assigned to distinct categories by their morphologies and neurochemical profiles. The variety of interneurons produced in foreign engraftment sites suggests that, although cerebellar progenitors are strictly committed to regional fates, even in ectopic locations their acquisition of different mature identities may still be influenced by environmental cues.

To address this question, GFP-tagged cells from the cerebella of P1 and P7 rats were injected in the ventricles of E15 rat embryos *in utero*, and their fate was examined 1 month post-transplantation. P1 donor cells engrafted in different places along the neuraxis, including neocortex, striatum, hippocampus, thalamus, hypothalamus, midbrain, and brainstem. Conversely, P7

cells were never found in the telencephalon, but they were mainly distributed in the midbrain and brainstem (**Figure 3A**). In line with previous findings, donor cells of both ages differentiated in glia, granule cells, and inhibitory interneurons, with larger fractions of neurons in the most caudal engraftment sites (Jankovski et al., 1996; Carletti et al., 2002). The phenotype of transplanted GABAergic interneurons was also evaluated by assessing their expression of markers specific for different categories: NeuN and calretinin (CR) for DCN interneurons, Pax-2 or neurogranin for Golgi neurons (**Figure 3B**), parvalbumin (PV) for ML interneurons (**Figure 3C**, Bastianelli, 2003; Singec et al., 2003; Leto et al., 2008; Rolando et al., 2010). Both P1 and P7 donors developed typical interneuron morphologies and expressed various markers in different host regions, confirming that they could acquire structural and neurochemical features distinctive of different categories of cerebellar interneurons even in ectopic positions. Among such donor-derived interneurons, 66.8% expressed PV (121/181 cells; $n = 10$ animals), 28.3% was CR-positive (49/173 cells; $n = 10$ animals), and 21.3% was NeuN-positive (37/174; $n = 10$ animals). Importantly, no grafted cells ever co-expressed PV and CR, confirming that these markers represent mutually exclusive phenotypic traits even in ectopically placed cells.

To understand whether the phenotypes acquired by heterotopically grafted interneurons were influenced by local cues, the neurochemical profiles of individual donor interneurons were compared to those of nearby host neurons. The vast majority PV-positive donor interneurons (112/121 cells) integrated in recipient regions where no endogenous PV-positive cells were present (**Figures 3D,G**). On the contrary, most of CR-positive (83.7%, out of 43/49 cells; **Figures 3E,G**) and NeuN-positive interneurons (96.5%, 109/113 cells; **Figures 3F,G**) were consistently intermingled with host cells expressing the same marker. These observations indicate that although cerebellar interneuron progenitors develop cerebellar-specific phenotypes in a cell-autonomous manner regardless of their ectopic location, at least some of their phenotypic traits may be influenced by local cues. This appears particularly evident for markers typical of DCN or GL interneurons, such as NeuN or CR, but not for PV, which is distinctive of ML interneurons. This suggests that the acquisition of DCN or GL interneuron identities is directed by external instructive information, whereas the ML phenotype can be acquired by executing some sort of default cell-intrinsic program.

CONCLUDING REMARKS

The findings reported in the previous sections indicate that distinct strategies sustain the generation of cerebellar GABAergic projection neurons and interneurons (**Figure 1**). All cerebellar GABAergic phenotypes derive from a single germinal layer, the VZ. Similar to other CNS sites, projection neurons are generated first, at the onset of neurogenesis, from progenitors that proliferate within the neuroepithelium. Their specification and differentiation are regulated by transcriptional programs expressed in specific subsets of VZ cells, distributed in discrete microdomains. As a consequence, the intrinsic fate potential of such cells is restricted at early ontogenetic stages and they acquire mature phenotypes essentially by unfolding cell-autonomous programs.

GABAergic interneurons derive from a single population of dividing cells that originate in the VZ, but continue their neurogenic activity in a secondary germinal site, the PWM. Transplantation experiments show that interneuron precursors are not restricted in their fate potential, but maintain the ability for generating the complete phenotypic repertoire up to the latest developmental stages. In addition, early postmitotic cells grafted to heterotopic/heterochronic recipients are still able to switch their fate and adopt host-specific interneuron identities. The latter feature suggests that upregulation of Pax-2, during the last cell

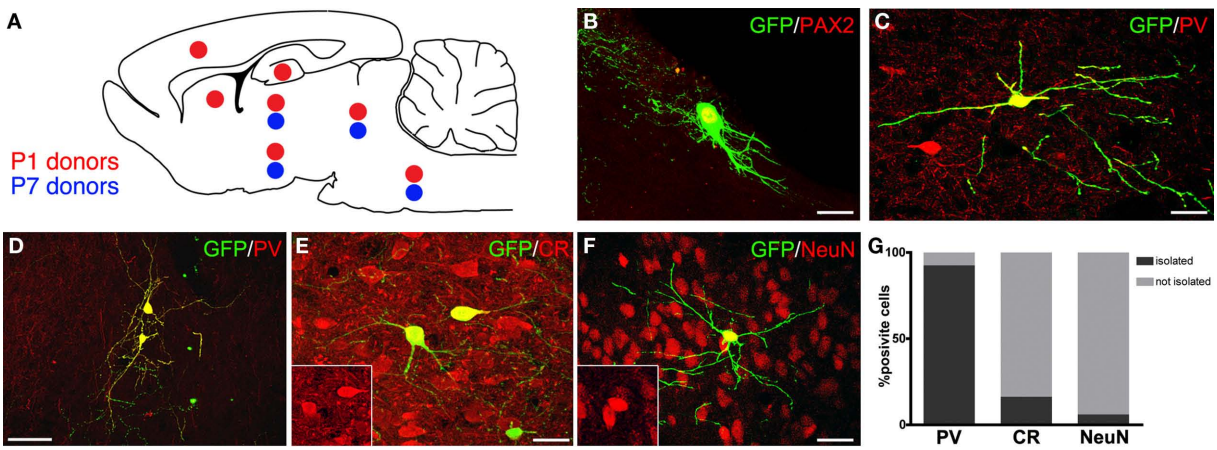

FIGURE 3 | Distribution and neurochemical profile of cerebellar progenitors heterotopically grafted to embryonic hosts *in utero*. (A) Distribution of P1 (red dots) and P7 (blue dots) GFP-expressing donor cells: P1 cells integrate widely along the neuroaxis, whereas P7 donors are found only in midbrain and brainstem. **(B,C)** Examples of GFP-positive cerebellar progenitors expressing Pax-2 and PV (red). **(D–F)** The ectopically positioned cells (green) express markers of cerebellar interneurons, such as PV [**(D)**, red], CR [**(E)**, red], and NeuN [**(F)**, red]. **(G)** Quantitative analysis of the distribution of transplanted interneurons expressing PV, CR, or NeuN in relation to the expression of the same markers in neighboring host neurons: the majority of PV-positive cells engraft in host regions where PV is not expressed **(D,G)**, whereas CR- **(E,G)** or NeuN-positive interneurons **(F,G)** are most often intermingled with host neurons expressing the same marker. Scale bars: in **(B,C,E,F)** = 20μm; **(D)** = 50μm.

cycle, marks the acquisition of the inhibitory interneuron identity, whereas the development of the distinctive traits of the different interneuron categories occurs in postmitotic interneurons under the influence of instructive environmental cues. The acquisition of site-specific traits by cerebellar interneurons that engrafted in extracerebellar sites is also consistent with this view.

Although this model of the genesis of cerebellar GABAergic neurons is corroborated by several lines of evidence, many fundamental questions remain unanswered. For instance, the lineage relationships linking projection neurons and interneurons are unclear. In this context, it is particularly important to define whether the same progenitors can generate both projection neurons and interneurons, or if these two categories derive from distinct pools of VZ cells. Another important issue refers to the nature and the source of the instructive cues present in the PWM milieu. We have recently proposed that specific signals may be issued by different cell populations that surround the PWM at subsequent ontogenetic stages (Leto et al., 2008, 2010). Although this idea is consistent with some preliminary observations, the mediators of the underlying signaling and their mechanisms of action remain obscure. Likewise, the composition and the neurogenic properties of the PWM itself have to be explored and characterized. Indeed, proliferating progenitors and young postmitotic interneurons pass through crucial decision points and undergo profound developmental changes being immersed in this environment. However, most of the cellular/molecular interactions that govern these processes and determine their outcome are still totally unknown. Finally, the reason why cerebellar interneurons are generated through such a peculiar mechanism has to be explained. The recent discovery of the prominent role played by the outer subventricular zone in the evolution of mammalian neocortex (Lui et al., 2011) highlights a potential adaptive function of secondary germinal sites, such as the cerebellar PWM, in providing a powerful ontogenetic mechanism to expand certain neuron populations and match their production rates to the requirements of rapidly evolving circuitries. All these issues, however, still await sound demonstration and, in spite of the considerable knowledge that we acquired about the developmental mechanisms of cerebellar interneurons, a long way has still to be run before we can really understand the fine nature of these processes.

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Cerebellum: links between development, developmental disorders and motor learning

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The study of the links and interactions between development and motor learning has noticeable implications for the understanding and management of neurodevelopmental disorders. This is particularly relevant for the cerebellum which is critical for sensorimotor learning. The olivocerebellar pathway is a key pathway contributing to learning of motor skills. Its developmental maturation and remodeling are being unraveled. Advances in genetics have led to major improvements in our appraisal of the genes involved in cerebellar development, especially studies in mutant mice. Cerebellar neurogenesis is compartmentalized in relationship with neurotransmitter fate. The Engrailed-2 gene is a major actor of the specification of cerebellar cell types and late embryogenic morphogenesis. Math1, expressed by the rhombic lip, is required for the genesis of glutamatergic neurons. Mutants deficient for the transcription factor Ptf1a display a lack of Purkinje cells and gabaergic interneurons. Rora gene contributes to the developmental signaling between granule cells and Purkinje neurons. The expression profile of sonic hedgehog in postnatal stages determines the final size/shape of the cerebellum. Genes affecting the development impact upon the physiological properties of the cerebellar circuits. For instance, receptors are developmentally regulated and their action interferes directly with developmental processes. Another field of research which is expanding relates to very preterm neonates. They are at risk for cerebellar lesions, which may themselves impair the developmental events. Very preterm neonates often show sensori-motor deficits, highlighting another major link between impaired developments and learning deficiencies. Pathways playing a critical role in cerebellar development are likely to become therapeutical targets for several neurodevelopmental disorders.

Keywords: cerebellum, learning, developmental, inferior olive, Purkinje neurons, genes, transcription factors

INTRODUCTION

Cerebellum plays critical roles in learning sensorimotor tasks (Manto, 2010). For instance, it is widely accepted that the olivocerebellar tract in one of the key pathways contributing to learning of new motor skills (Ito, 2006). However, although the involvement of cerebellar circuits in motor learning is known to be critical, their precise role in the acquisition and storage of new motor abilities or rather in the performance of the acquired motor skills is still a matter of debate. Several authors have pointed that attention should be paid to functional states (as opposed to neural sites) able to generate motor learning in mammals (Delgado-García and Gruart, 2002). Permanent or temporary disconnection of a given nodal center in a neural circuit will not determine the whole functional state of the involved circuit and the transformation of neural signals occurring at the different neural centers included in the circuit (Harvey et al., 1993; Delgado-García and Gruart, 2002).

There is currently a growing awareness that neurodevelopmental disorders are associated with cerebellar deficits and learning impairments. Still, the molecular mechanisms of the cerebellar defects remain poorly understood in many cases. Cerebellum is likely to become a major platform to investigate how development and learning interact in mammals. Indeed, cerebellar circuits are modular and stereotyped from the morphological standpoint and provide thus a structure of choice to investigate the relationships between regional developmental defects and learning, especially from the anatomical/functional point of view. Moreover, marked morphological changes still occur after birth, allowing the detailed assessment of developmental abnormalities with various techniques and their phenotypical impact after the pregnancy. In addition, developmental studies have revealed that the cerebellum evolves in successive waves of progenitors proliferation/migration throughout the embryonic and postnatal phases. The possibility to act selectively on these waves opens new therapeutic doors.

This review covers recent advances in the understanding of the gene networks contributing to cerebellar development, discusses the impact of very premature birth upon cerebellar development and underlines the critical steps of the development of the olivocerebellar tract. Potentially clinically relevant discoveries are highlighted.

THE KEY-FEATURES OF CEREBELLAR DEVELOPMENT

Because the circuits of the cerebellum are unique in their morphology, the mechanisms of cerebellar neurogenesis are a subject

of intense investigation (Carletti and Rossi, 2008). Neuronal/glial migrations as well as dendritogenesis are fundamental processes leading to functional cerebellar microcircuits being effective for plasticity and learning. Interestingly, the anatomy of the cerebellum with a midline vermis and two hemispheres located laterally is highly conserved from rodents to human, suggesting that the analysis of the development in rodents should provide direct relevant informations in human, including for cerebellar malformations.

The major features of cerebellar development can be briefly summarized as follows. Neuronal populations are generated in a sequential manner. The inhibitory interneurons emerge from the ventricular zone and the glutamatergic neurons are generated by the rhombic lip (Carletti and Rossi, 2008). In mouse, the glutamatergic and gabaergic neurons in nuclei are produced first, followed by Purkinje neurons. It is established that gabaergic interneurons of the cerebellar cortex originate from a ventricular zone progenitor (Leto and Rossi, 2011). After generation of cerebellar nuclei, the external granular layer is formed from precursors of granule cells originating from the rhombic lip. Granule cells will migrate to form the internal granular layer. It is interesting to note that these events occur at the third trimester of development in human (see also below the impact of very premature birth upon cerebellar development). Survival and maintenance of Purkine neurons and granule cells is dependent on the antiapoptotic protein Lifeguard, which is highly expressed in the cerebellum and is strongly upregulated during postnatal brain development (Hurtado de Mendoza et al., 2011). Lifeguard antagonizes the FAS pathway. FAS receptors tune neuronal survival following trophic factors deprivation (Raoul et al., 2000). Lifeguard affects cerebellar size, internal granular layer thickness, and Purkinje cell development, suggesting that lifeguard could participate in the pathogenesis of various human cerebellar disorders characterized by cerebellar atrophy. Glutamatergic unipolar brush cells migrate to the internal granular layer. Whereas the ventricular zone will lose its progenitors at late embryogenic stages, the rhombic lip remains active until postnatal period.

CEREBELLUM, HORMONES, AND NEUROSTEROIDOGENESIS

The relationship between circulating hormones and cerebellar development is well demonstrated. In particular, thyroid hormone plays a critical role in brain development (Koibuchi, 2008). The thyroid hormone receptor is a ligand-regulated transcription factor binding to a specific DNA sequence called thyroid-hormone-responsive element. The receptor recruits various coregulators such as coactivator and corepressor in a ligand-dependent manner to modulate the transcription of target genes (Koibuchi, 2008). It may also interact with other nuclear receptors such as Rora (retinoic-acid-related orphan receptor alpha; see below) whose expression is regulated by the thyroid hormone during the first postnatal two weeks.

In perinatal hypothyroidism, the growth and branching of Purkinje cell dendrites are greatly reduced, there is a reduction of synapses between granule cells and Purkinje neurons, migration of granule cells to the internal granule cell layer is delayed and synaptic connectivity within the cerebellar cortex is deficient (Nicholson and Altman, 1972; Koibuchi et al., 2003, 2008; **Figure 1**). Thyroid deficient rats show a persistence of synaptic sites of climbing fibers for a longer time (see section on the remodeling of the olivocerebellar projection) along with an underdevelopment of cerebellar glomeruli (Hajós et al., 1973). Performance of the hypothyroid animals is impaired in tests of adaptive behavior.

The discovery that Purkinje neurons possess steroidogenic enzymes and produce progesterone from cholesterol in the neonatal period has provided a link between steroidogenesis and development of cerebellar circuits (Sakamoto et al., 2001). Concentrations of progesterone and allopregnenolone are high in the cerebellum during the post-natal life (Tsutsui, 2008). The group of Tsutsui has demonstrated that progesterone promotes dendritic growth and spine formation in Purkinje neurons (Sakamoto et al., 2001). Neurosteroids have a direct effect upon synaptogenesis during the neonatal development. Formation of cerebellar circuitry is dependent on a local steroidogenesis, acting through neurotrophic factors such as BDNF (Sasahara et al., 2007). This emphasizes a potential novel bridge between neurosteroidogenesis and motor learning, with possible therapeutical implications in developmental disorders.

THE ENGRAILED-2 GENE

The engrailed (En) homeobox transcription factor family is critical for the patterning of cerebellar lobules and for Purkinje cells protein stripes (Kuemerle et al., 1997). The En1/2 regulates the targeting of mossy fiber systems to subsets of cerebellar lobules, showing a main role for the afferent topography in the cerebellar circuitry (Sillitoe et al., 2010). Initially, the En1/2 mRNA/protein are expressed in the ventricular zone. During early post-natal cerebellogenesis, En1/2 are expressed in spatially restricted patterns in most cell types. It is plausible that En1/2 are implicated in neurodevelopmental disorders such as autism spectrum disorder (see also below). Indeed, mutant mice EN2−/− show neurobehavioral and neurochemical deficits suggestive of autism spectrum disorder (Cheh et al., 2006).

MATH1

The specification and differentiation of glutamatergic lineages is dependent upon Math1, a transcription factor of the bHLH class. Math1 is critical for the proper development of the granular layer of the cerebellum. Mice deficient in Math1 show a loss of glutamatergic neurons in cerebellar nuclei, a loss of external granular layer and unipolar brush cells. In addition, Math1 null embryos lack interneurons giving rise to the spinocerebellar and cuneocerebellar tracts (Bermingham et al., 2001).

PTF1A AND ASCL1

Cerebelless mutants have a deficit in the transcription factor Ptf1a (pancreatic transcription factor 1a). They show a lack of Purkinje cells and gabaergic interneurons. It has been demonstrated that climbing fiber neurons are derived from the Ptf1a domain (Yamada et al., 2007). In Ptf1a null mutants, immature climbing fiber neurons cannot migrate or differentiate, causing a failure in the formation of the inferior olivary nucleus. Ptf1a is also involved in the control of fate and survival of neurons during

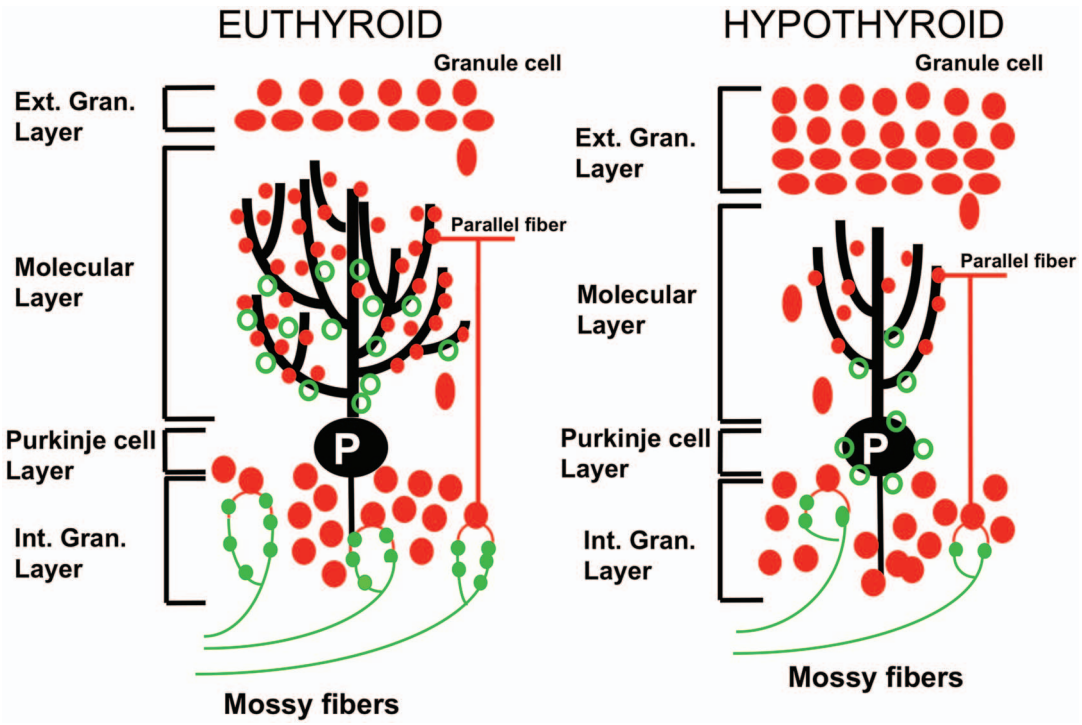

FIGURE 1 | Effect of perinatal hypothyroidism on neurogenesis and differentiation of the cerebellar cortex. In the hypothyroid cerebellum (right panel), disappearance of the external granular layer is delayed consequently to the retarded proliferation and migration of granule cells to the internal granular cell layer. The arborisation of the dendrites of Purkinje neurons (P) is decreased. The connectivity between parallel fibers (pf; red closed circles) and Purkinje cells is reduced. The synaptic connections between mossy fibers (mf; green closed circles) and granule cells are decreased as compared to the euthyroid condition. Green open circles: climbing fibers synapses. Adapted from Koibuchi et al. (2003).

development. In human, mutations of Ptf1a are associated with cerebellar agenesis (Sellick et al., 2004).

Ascl1 directs ventricular neuroepithelium progenitors toward inhibitory interneuron fate and suppresses the astrocytic differentiation (Grimaldi et al., 2009). Mice lacking Ascl1 in the cerebellum exhibit a major decrease of cerebellar interneurons and an imbalance between oligodendrocytes and astrocytes (Sudarov et al., 2011).

RORA (RETINOIC-ACID-RELATED ORPHAN RECEPTOR ALPHA) GENE

Rora is a transcription factor encoding a retinoid-like nuclear receptor which is highly expressed in the cerebellum (Hamilton et al., 1996). Rora belongs to the steroid-thyroid hormone receptor superfamily (Koibuchi, 2008). Its endogenous ligand is cholesterol which is abundantly present in each cell. Therefore, Rora acts as if it is a constitutively active nuclear receptor (Koibuchi, 2008). It was initially thought that Rora was exclusively expressed in neurons, but recent data show that it is also expressed in glial cells especially in astrocytes (Journiac et al., 2009). Rora plays a pivotal role in the development of the cerebellum, olfactory bulb, and retina (Jetten, 2009). However, its functions extend beyond development. For instance, Rora also protects neurons against oxidative stress and shows an anti-inflammatory action by inhibiting the NF-Kappa-B pathway (Delerive et al., 2001; Boukhtouche et al., 2006).

The autosomal recessive *staggerer* mutation is associated with a severe degeneration of Purkinje neurons with a nearly total absence of granule cells at the end of the first postnatal month (Landis and Sidman, 1978). The homozygous mouse $Rora^{sg}/Rara^{sg}$ is highly ataxic, whereas the heterozygous mouse $Rora^{+}/Rara^{sg}$ appears phenotypically normal, showing disabilities during challenging tasks.

REELIN AND CEREBELLAR DEVELOPMENT

The external granular layer promotes Purkinje cell migration by secreting reelin (RELN), an extracellular matrix component attracting or repealing precursors and axons during development, acting as an extracellular signaling molecule. Reelin deficient mice (Reeler) show a severe cerebellar hypoplasia. They exhibit Purkinje cell migration defects and cerebellar nuclei are impaired. Foliation is absent. Reelin continues exerting activities beyond birth. It modulates long-term potentiation and is thus involved in learning (Beffert et al., 2004). In the adult brain, Reelin regulates structural and functional properties of synapses. Its overexpression may increase markedly the long-term potentiation responses and it has been proposed that Reelin controls developmental processes remaining active in the adult brain (Pujadas et al., 2010). In human, reelin might be implicated in some forms of lissencephaly (due to neuronal migration defect) and could contribute to the pathogenesis of autism (**Figure 2**).

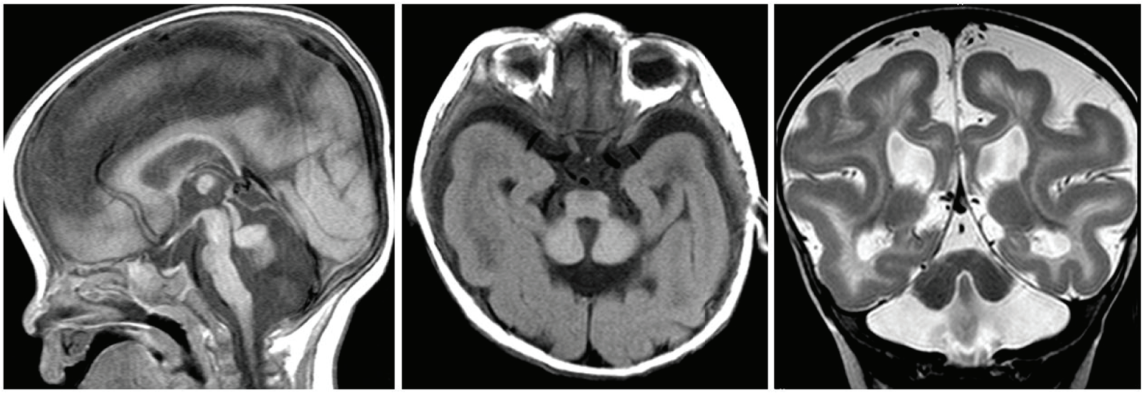

FIGURE 2 | RELN mutation. Sagittal (left panel) and axial T1- (middle panel), and coronal T2-weighted (right panel) images showing a very small afoliar vermis and cerebellar hemispheres. The severe hypoplasia of the cerebellum is associated with lissencephaly, cerebral hemispheres showing thickened cortex and simplified gyral pattern. Hippocampal hypoplasia and malrotation are part of the specific MRI features.

THE CHEMOKINE RECEPTOR 4 (CXCR4)-CHEMOKINE LIGAND 12 (CXCL12) SYSTEM

Chemokines and their receptors are determinant in cell migration and in organogenesis (Zou et al., 1998). CRXC4 and CXL12 mutant mice show proliferating granule cell progenitors located in deeper location (Zou et al., 1998). Down-regulation of CXCR4 causes an inward radial migration of granule cells precursors. The chemoattractant SDF-1α and its receptor CXCR4 attract the cerebellar granular neuronal precursors to the outer external granular layer and promote an increase of the sonic hedgehog mitogenic effect (see next section).

SONIC HEDGEHOG AND CEREBELLAR DEVELOPMENT

Sonic hedgehog is highly expressed in the cerebellum (Vaillant and Monard, 2009). Sonic hedgehog is a morphogenetic factor which is a masterplayer in cerebellar patterning and foliation (Vaillant and Monard, 2009). Indeed, sonic hedgehog controls the proliferation of progenitors in the cerebellum (**Figure 3**). Sonic hedgehog pathway involves the GLI family of transcription factors. The binding of sonic hedgehog to the transmembrane receptor Patched 1 triggers a cascade of events tuning cAMP production (DeCamp et al., 2000). A link between cholesterol metabolism, sonic hedgehog and cerebellar development has been established. Indeed, cholesterol deficiencies are associated with defects in the sonic hedgehog signaling (cholesterol is an activator of sonic hedgehog) and cause cerebellar malformations (Lanoue et al., 1997). Hypoplasia of the cerebellum is classically associated with the Smith–Lemli–Opitz syndrome, an autosomal recessive syndrome with multiple congenital malformations, which is due to defects in cholesterol homeostasis (Dehart et al., 1997). Sonic hedgehog exerts critical mitogenic functions (Vaillant and Monard, 2009). For instance, sonic hedgehog stimulates very strongly the proliferation of cerebellar granular neuronal precursors through the induction and repression of cell cycle regulators genes (Wechsler-Reya and Scott, 1999). Blocking GLI2 causes a failure in the development of cerebellar granular neuronal precursors, ending in cerebellar hypoplasia (Corrales et al., 2004). In addition, sonic hedgehog contributes to cerebellar cortex development by promoting Bergmann glia proliferation and thus contributing to the migration support (Corrales et al., 2006; Vaillant and Monard, 2009). While delection of sonic hedgehog leads to an absence of foliation and underdevelopment of the cerebellum, the sonic hedgehog mutants show a larger cerebellum, even with an extralobule, demonstrating how sonic hedgehog determines cerebellar morphology and shape (Corrales et al., 2006). Cyclins D1 and D2 are transcriptional targets of sonic hedgehog. Delection of cyclin D2 is associated with a mild hypoplastic cerebellum (Huard et al., 1999).

The extra-cellular matrix undergoes a substantial remodeling during cerebellar development, providing cues for division or differentiation according to its content (Vaillant et al., 1999; Vaillant and Monard, 2009). Laminin and heparan sulfate proteoglycans increase the mitogenic effects of sonic hedgehog (Rubin et al., 2002).

Sonic hedgehog pathway is also controlled by negative regulators, such as PACAP (pituitary adenylate-cyclase activating polypeptide). Knock-out mice for PACAP show an overactive sonic hedgehog pathway with enlargement of the external granular layer (Nicot et al., 2002). Sonic hedgehog is also deactivated by FGF-2, which triggers the differentiation of neural precursors of granule cells (Fogarty et al., 2007).

Sonic hedgehog is implicated in the formation of medulloblastoma, an aggressive tumor of the cerebellum. Mutations of the PTC receptor lead to an overactivation of sonic hedgehog (Vaillant and Monard, 2009). The overexpression of sonic hedgehog in neural progenitors of the cerebellum causes a medulloblastoma (Weiner et al., 2002). GLI1 expression is markedly increased in a subgroup of patients developing a medulloblastoma (Ferretti et al., 2008). Development of sonic hedgehog antagonists might be considered to manage this tumor.

THE pcd MODEL

Purkinje cell degeneration (pcd), an autosomal recessive mutation in the mouse, causes the postnatal death of nearly all cerebellar Purkinje cells during the third and fourth postnatal

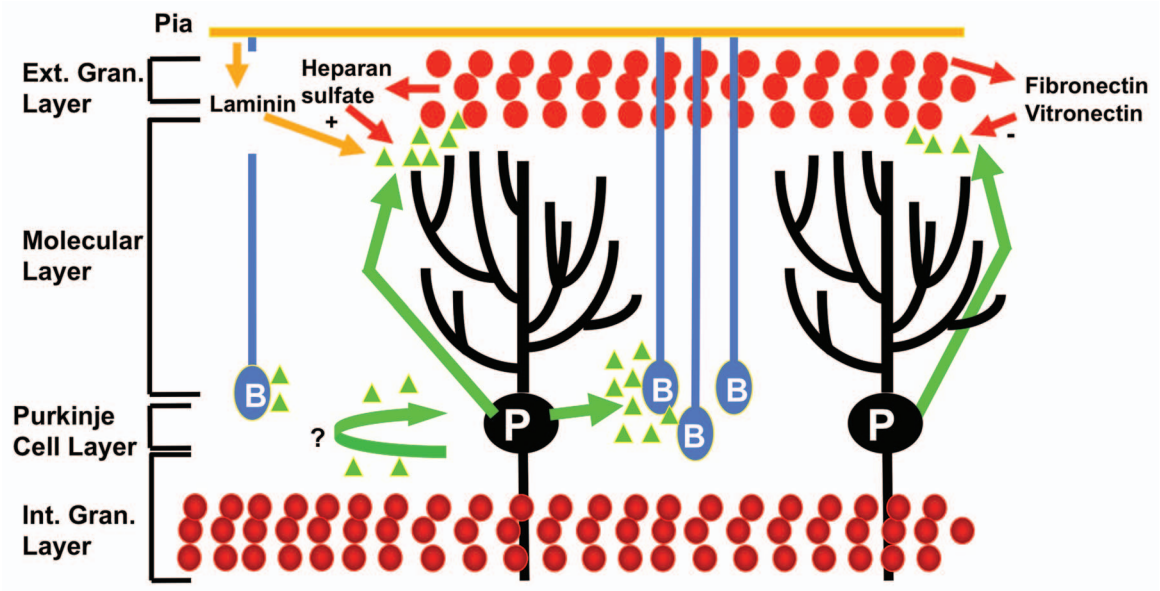

FIGURE 3 | Effects of sonic hedgehog (represented by green triangles) on cerebellar development. At the postnatal period, sonic hedgehog is secreted by the Purkinje neurons (P), triggering mitosis in the outer external granular cell layer (cerebellar granular neuronal precursor cells illustrated by red circles), Bergmann glia (B) differentiation, and also possibly Purkinje cells maturation. The promitogenic effect on cerebellar granular neuronal precursor cells is synergized by cooperative modulators such as laminin and heparin sulfate, whereas fibronectin and fibronectin act as negative modulators. Mature granular cells are represented at the level of the internal granular layer (bottom part of the figure). Adapted from Vaillant and Monard (2009).

week (Landis and Mullen, 1978; Sotelo and Alvarado-Mallart, 1986). This strain has undergone an extensive investigation (Wang and Morgan, 2007). Several independent phenotypic alleles have been identified with mutations in the Nna1 gene. The model is characterized by a moderate ataxia developing between three and four weeks of age. The degeneration of Purkinje neurons begins around 18 days and progresses quickly over two weeks. At about four months, most of the Purkinje neurons have degenerated. Before Purkinje cells start degenerating, they appear to receive all their synaptic contacts (Landis and Mullen, 1978). The mice show impaired eyeblink conditioning and abnormal spatial navigation learning.

MUTATIONS OF HERC GENE

Proteins with HECT domains act as ubiquitin ligases. Recently, it has been shown that mutations in the highly conserved N-terminal RCC1-like domain of the HERC1 protein cause a progressive Purkinje cell loss leading to severe ataxia with reduced growth and lifespan in homozygous mice aged over two months (tambaleante mutant mice; Mashimo et al., 2009). Activities of the proteins encoded by the HERC gene family are critical in a number of important cellular processes such as cell cycle, cell signaling, and membrane trafficking. It is now established that they play a key contribution in the physiology of Purkinje neurons.

REMODELING OF THE OLIVOCEREBELLAR PROJECTION AND COMPENSATION FOLLOWING AN INJURY TO THE CEREBELLUM

The olivocerebellar tract plays a major role in the control and regulation of the striated muscle system, although non-exclusively. Several authors have pointed out its contribution in motor learning (Apps and Lee, 2002). After leaving the inferior olive nucleus, the olivocerebellar axon crosses the midline between the bilateral olives and enters the cerebellum via the contralateral inferior cerebellar peduncle. The axon ramifies into thick branches and thin collaterals. Climbing fibers are characterized by strong synaptic connections to Purkinje cells with an exclusive one-to-one relationship (Eccles et al., 1966; Sugihara, 2006). There is an average of 6.6 climbing fibers per axon in adult rats (Sugihara et al., 2001). Climbing fibers originating from one axon send thin collaterals to cerebellar nuclei and the granular layer, and end in a narrow longitudinal band called microzone (Sugihara et al., 1999). During normal development, the olivocerebellar axon undergoes a remodeling to reach the final configuration (Sugihara, 2006). Studies in newborn rats at postnatal days 4–7 (P4–P7) have shown that there are many more nuclear collaterals and swellings as compared to adult olivocerebellar axons, with swellings more densely packed (Sugihara, 2006). The basic organization of the olivocerebellar tract is reached at P4–P7, since the paths and destinations of the collaterals (cerebellar nuclei and the granular layer) are nearly identical with the adult configuration. The microzonal distribution is already present, suggesting that the pattern of projection is determined by a molecular recognition mechanism that is active from the beginning of the formation of the olivocerebellar projection. (Sugihara, 2005, 2006). Nevertheless, immature—so-called "nest"—climbing fibers can be distinguished from adult climbing fibers by their morphological aspect. As pointed by Sugihara, nest-type climbing fibers are characterized by a dense aggregation of swellings around the soma of a Purkinje cell with a few long fibers extending outside the aggregation. At the nest stage,

Purkinje cell shows a single apical dendrite beginning to grow while other short protrusions in the soma vanish. The terminal arbor of the climbing fiber moves upwards to cover the stem of the apical dendrite ('capuchon' or 'hood') and to cover the dendritic tree proximally (mature climbing fiber) over the next 10 days (Mason et al., 1990). Before the nest period, the terminal portion of olivocerebellar axons (still called 'climbing fibers') creeps between Purkinje cells aligned in the multiple-cellular layer. They are designated as "creeper" (Chedotal and Sotelo, 1993). The "transitional type" has a terminal arbor with features intermediate between those of the nest and the creeper types (Sugihara, 2005). Transitional-type terminal arbors are characterized by thin terminal fibers with moderate aggregations of swellings around Purkinje cells. Other types include the "atrophic nest," with less swellings and smaller harbors as compared to the full nest. It is assumed that the remodeling of climbing fibers is mainly due to local factors in the cerebellar cortex, rather than an intrinsic programming (Sugihara, 2006). Because both the granular layer and the nest-type climbing fibers emerge early in the vermis, it has been suggested that the granule cells and their parallel fibers might subserve the remodeling of nascent climbing fibers.

Another major event is the decrease in the number of climbing fibers (Crépel et al., 1976). The elimination of supernumerary climbing fibers will reduce the ratios climbing fibers/single axons from 100 (creeper stage) at P5 up to about seven for the adult stage, indicating an important retraction or pruning phenomenon (Sugihara, 2006). Glia likely controls this mechanism (Awasaki and Ito, 2004). The change from a multiple innervation of Purkinje cells by climbing fibers to a monoinnervation is observed during the first two and three postnatal weeks in rodents (Scelfo and Strata, 2005). A competition occurs between multiple climbing fibers at the level of the soma and the proximal dendrite of the Purkinje neuron, with elimination of climbing fibers except one (Hashimoto and Kano, 2003). There is a general agreement that climbing fiber remodeling from the creeper to the nest type is a major event for the synaptic maturation and the initial selection process (Sugihara, 2006).

Interestingly, targeted deletion of mGluR1 gene causes both developmental defects and dysfunction of the cerebellum. mGluR1−/− mice exhibit abnormal regression of multiple climbing fiber innervation, impaired long-term depression, and abnormal motor coordination (Ichise et al., 2000). This illustrates that a receptor is involved both in developmental processes and synaptic plasticity, providing a bridge between these neurobiological processes.

The changes in the olivocerebellar system reported above are fundamental events linking development and learning. They are a prerequisite to reach functional olivocerebellar units. Cerebella which have been degranulated by post-natal X-ray exposure show a multiple innervation of Purkinje neurons by climbing fibers which is maintained (Mariani et al., 1990).

Following unilateral transection of climbing fibers early in development, the contralateral inferior olive degenerates. New axons emerging from the unaffected inferior olive reach the denervated hemicerebellum to partly recreate the olivocerebellar circuit (Sugihara et al., 2003). This reinnervation is associated with an improvement in motor abilities (Dixon et al., 2005). It has been shown that the amount of transcommissural reinnervation is directly correlated with spatial performance (Willson et al., 2007). Partial circuit repair confers a significant benefit from the functional point of view. The neonatal period might be particularly suited for procedures aiming to assist, promote, or guide reinnervation.

Innervation of multiple climbing fibers onto Purkinje cells may persist in adulthood in protein kinase C-gamma mutant mice (Chen et al., 1995). These mutant mice exhibit an impaired motor coordination but both eyeblink conditioning and cerebellar long-term depression are preserved. This latter phenomenon had been considered as a prerequisite for cerebellar motor learning, but recent studies show no motor learning impairment in mutant mice lacking parallel fiber-Purkinje neuron synapses long-term depression, demonstrating that this is not an essential step for cerebellar motor learning (Schonewille et al., 2011).

Compensatory mechanisms occur also for genetically determined mutant mice such as Lurcher, which represents a natural model of olivocerebellar degeneration (Cendelín et al., 2009). They carry a mutation of the glutamate receptor delta2-subunit gene which is predominantly expressed by Purkinje neurons (Zuo et al., 1997). Homozygous mutants are not viable. A complete postnatal loss of Purkinje cells and a secondary decrease in the number of granule cells/inferior olive neurons occurs. Granule cells and neurons of the inferior olive degenerate due to the loss of Purkinje neurons (Wetts and Herrup, 1982). The lack of proliferation of granule cells precursors is primarily due to the deficit in the mitogenic effects of sonic hedgehog (Sajan et al., 2010). The degeneration of Purkinje neurons is complete at P90. Young adult heterozygous Lurcher mice constitute an excellent model to investigate the role of the cerebellar cortex in motor performance—including the acquisition of new motor abilities—due to the early postnatal degeneration of nearly all Purkinje and granular cells. They show compensatory adjustments in the correlations of firings between cerebellar nuclei and brainstem nuclei such as red nuclei (Porras-García et al., 2010).

NEURODEVELOPMENTAL DISORDERS

Thanks to novel perinatal neuroimaging techniques, cerebellar malformations are increasingly recognized in the fetal period (Bolduc et al., 2011). A typical example is Joubert syndrome. The disorder presents with developmental delay, hypotonia, impaired respiration, abnormal eye movements, and ataxia (Joubert et al., 1969). Motor learning is strongly impaired. The "Molar tooth sign" (deep interpeduncular fossa, enlarged superior cerebellar peduncles which are more horizontally oriented and hypoplastic cerebellar vermis) is very suggestive. Joubert syndrome is associated with mutations of genes encoding components of the primary cilia. Interestingly, primary cilia are determinant for sonic hedgehog signal transduction (Vaillant and Monard, 2009). Disruption of primary cilia formation blocks the proliferation of neural progenitors of granule cells mediated by sonic hedgehog (Spassky et al., 2008).

Another disorder clearly associated with learning disabilities is rhombencephalosynapsis, a malformation of the hindbrain characterized by fusion of the cerebellar hemispheres and dentate nuclei. It is assumed that the disorder is due to a failure of dorsal

patterning at the midbrain-hindbrain boundary (Pasquier et al., 2009). Other cerebellar malformations which are encountered in daily practice include Dandy–Walker malformation, vermis hypoplasia, mega cisterna magna, and posterior fossa retrocerebellar cyst. Sonic hedgehog might also be involved in the pathogenesis of Dandy–Walker malformation through a contribution of Zinc finger transcription factors which modulate the sonic hedgehog pathway (Aruga, 2004).

Autism spectrum disorders are characterized by difficulties in communication, social skills, and repetitive behavior. Cerebellar networks might be critically involved in the pathogenesis of autism. An immune dysfunction with local inflammation contributes to the pathogenesis of autism (Wei et al., 2011). The expression of IL-6 is increased in the cerebellum of autistic patients. IL-6 impacts upon the development of the cerebellum, impairing neural cell adhesion, migration, and causing an excessive formation of excitatory synapses (Wei et al., 2011).

Recent studies underline the a high prevalence of neurologic, developmental, and functional disabilities including motor, cognitive, language, and social-behavioral deficits in children with cerebellar malformations (Bolduc et al., 2011). The associated supratentorial anomalies, chromosomal findings, and malformations affecting the cerebellar vermis are independent predictors of neurodevelopmental disabilities. Furthermore, the associated supratentorial anomalies and chromosomal findings are predictive of cognitive impairment, gross and fine motor delay. Moreover, malformations of the vermis are predictors of gross motor delays (Bolduc et al., 2011). There is a clear need for early intervention services aiming to improve the daily care of these patients, especially when gross motor impairments and learning deficits are on the forefront of the syndrome.

IMPACT OF VERY PREMATURE BIRTH UPON CEREBELLAR DEVELOPMENT

Very premature birth (less than 32 weeks of gestation) may impact upon the cerebellar development, with consequences upon cognition and behavior. The prevalence of preterm birth is estimated to 5–9% (Spreen et al., 1995). The prevalence of very preterm birth is about 0.9%. Several disabilities have a higher incidence in preterms, such as developmental delay, learning deficits, cerebral palsy, and sensory impairments. The most preterm infants often show the most severe disabilities. Neonates following a gestation of less than 32 weeks are developmentally immature and their brain is particularly susceptible. Several types of brain injury have been reported, such as periventricular hemorrhage and hemorrhagic infarction, periventricular leukomalacia, and diffuse white matter injury. The injury of the white matter is now detected in the majority of very premature neonates (Dyet et al., 2006). These findings may occur in the cerebellum as well, and are often associated with a development delay of the posterior fossa structures, often reduced in size (**Figure 4**). There is a correlation between the MRI findings and the psychomotor outcome (Drobyshevsky et al., 2007). Although Purkinje neurons and neurons of the cerebellar nuclei develop early *in utero*, the granule cells show developmental/migrational processes occurring during the last trimester. Therefore, a hemorrhage in the cerebellum will impair these processes. Indeed, premature neonates are at risk for cerebellar hemorrhage. The hemorrhage may be unilateral and associated with extra-cerebellar lesions (Limperopoulos et al., 2005). Neonates with a cerebellar hemorrhage show a slower cerebellar growth. The mortality is higher in this case. It is estimated that the maximal cerebellar vulnerability is between 26 and 28 weeks (Limperopoulos et al., 2005). Cerebellar development may also be affected by extra-cerebellar lesions, as a result of loss of trophic actions exerted by pathways projecting to the cerebellum. Contralateral cerebellar atrophy can also occur as the result of a supratentorial lesion.

The likelihood to present sensori-motor combined with cognitive disabilities is higher in case of very premature birth. It has been shown that there is higher risk to develop psychiatric/behavioral disorders later in life (Byrne et al., 2007). Premature children are at higher risk to develop autism or autism spectrum disorders (Limperopoulos et al., 2008). There is growing evidence that the cerebellum plays key-roles in the pathogenesis of autism, as pointed out earlier. Individuals born very preterm tend to have lower IQs as compared to matched controls and often show trends toward academic difficulties in adolescence and young adulthood (Allin et al., 2008; Narberhaus et al., 2009). Volumetric studies performed in adolescents with a history of very preterm birth have shown reduced cerebellar size, especially in the lateral lobes which are known to participate in cognitive operations (Allin et al., 2005). Regional gray matter volumes are reduced in sensorimotor and parieto-occipital regions.

The recent fMRI study of visuo-perceptual learning processing in adults born very preterm has shown an increased BOLD signal response as compared to controls during the recognition phase in the right cerebellum and in the anterior cingulate gyri (Narberhaus et al., 2009). However, this disappeared after controlling for the probability of the absolute amount of gray matter in the hippocampus. The authors have hypothesized that cerebellar atrophy associated with hippocampal atrophy, affect cerebellar activation only when hippocampal size is not accounted for. It seems that very preterm individuals have a less efficient neural processing.

It is currently unclear how very preterm birth impacts upon epigenetic factors. Still, this is a question of great interest. Certain molecules, like BDNF, play major roles in cerebellar development and are involved in the recovery from hypoxia in the neonatal period (Almli et al., 2000).

THE EXAMPLE OF A COMMON DEVELOPMENTAL DISORDER: DEVELOPMENTAL DYSLEXIA

Developmental dyslexia can be defined as "a disorder in children who, despite conventional classroom experience, fail to attain the language skills of reading, writing, and spelling commensurate with their intellectual abilities" (according to the World Federation of Neurology). Its prevalence is very high, from 3% to 10% of learning disabilities (Bishop and Snowling, 2004). Skills in children are very heterogeneous. There is evidence that dyslexia is associated with mild clumsiness and deficit in fine motor skills (Nicolson and Fawcett, 2011). Dyslexia is associated with difficulties in making skills automatic (Nicolson and Fawcett, 1990). Most patients exhibit balance difficulties.

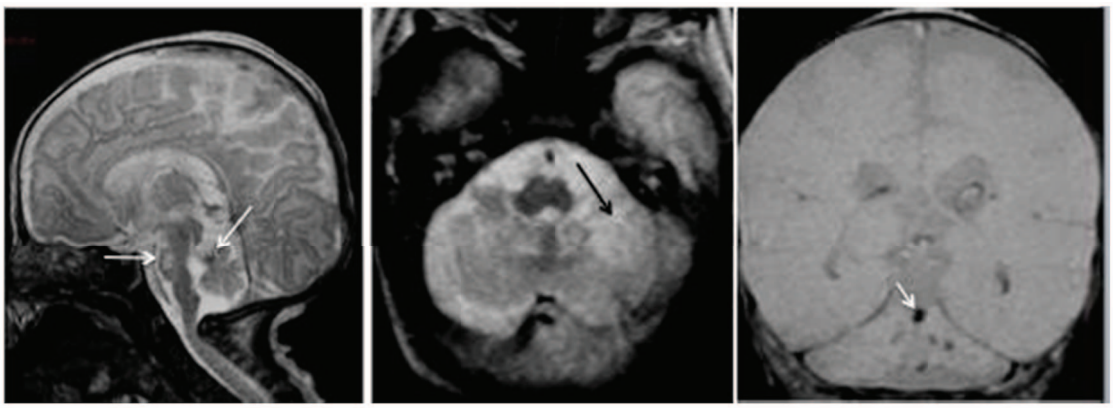

FIGURE 4 | Premature baby born at 27 gestational weeks and MRI performed at 39 gestational weeks. Left panel: the sagittal T2-weighted image shows reduced size of the posterior fossa (vertical tentorium), small vermis, and pons with a hemorrhagic dark spot on the anterior vermis (left arrow) and bright signal in the pons (right arrow) corresponding to hypoxic ischemic necrosis. Middle panel: the axial T2-WI shows the ischemic necrosis of the left cerebellar hemisphere (arrow) appearing bright. Right panel: the coronal T2 gradient-echo image shows multifocal dark signals in the vermis (arrow) and the left cerebellar hemisphere corresponding to hemorrhagic foci.

The hypothesis of a cerebellar dysfunction is straightforward given the major role played by the cerebellum in learning, automaticity of skills and its contribution to language (Nicolson et al., 1995). An ontogenetic framework has been proposed to explain how cerebellar differences at birth cause a range of difficulties in children (Nicolson and Fawcett, 2011). The language-related regions of the cerebellum, namely lobules VI and VIIB, would be affected in dyslexia. Procedural learning circuits involving the cerebellum would be primarily affected. An abnormal development of the brain, including the cerebellum, during the gestation has been proposed. Genetic investigations will very likely lead to the discovery of the mechanisms leading to some of these learning disabilities.

CONCLUSION

In this mini-review, we have summarized recent advances in our understanding of the molecular mechanism governing cerebellar development. We have discussed the complex interactions between cerebellar development and motor learning. The identification of several pathways which are potential targets for novel therapies in the future, such as cerebellar neurosteroidogenesis, En1/2, Math1, Ptf1a, Rora, or sonic hedgehog, is now bringing hope in a field which has often remained neglected because of a lack of understanding of the molecular events leading to the malformations. There is still a growing need to identify new targets, since neurodevelopmental disorders are heterogeneous and will impact upon the whole life of patients in most cases. Protecting the developing cerebellum—a concept which could be called cerebelloprotection—is now attracting the interest of the scientific community, especially with discoveries of the roles of the cerebellum in cognitive skills. Very preterm neonates are an example of a population of patients at risk and which could benefit from neuroprotecting actions.

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