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Molecular events directing the patterning and specification of the cerebellum

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SUMMARY

The vertebrate brain is a remarkably complex anatomical structure which contains diverse subdivisions and neuronal subtypes with specific synaptic connections that contribute to the complexity of its function. The neural tube (the primordial brain) has to be progressively regionalized by means of precise control of the spatial and temporal arrangement of an orchestrated cocktail of genes. These will regulate inter- and intracellular signals driving a proper molecular patterning and specification of the distinct brain subdivisions, and thus will generate the structural basis of complexity and cellular diversity which characterize the brain.

The present revision focuses on the main molecules involved during early development of the vertebrate cerebellum, the most rostral and dorsal structure of the hindbrain. We will survey the literature related to the early molecular mechanisms arising from the isthmus to pattern the caudal midbrain and rostral hindbrain primordia. The isthmus retains morphogenetic properties to further refining these subdivisions. Once the patterning of the cerebellar anlage is established, further molecular events (coming from the ventricular side and the rhombic lip) will specify the diverse neural cell population and the fine-tuning of the stereotyped morphology and layers of the cerebellum.

Finally, we will discuss the combination of molecular genetics (gene expression pattern maps) and modern neuroanatomy (based on immunohistochemistry and highly sensitive neuroimaging), which 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 cerebellum.

Key words: Fgf8 – Secondary organizer – Midhindbrain junction – Cerebellum – Mesencephalon – Rhombencephalon – Morphogenesis

THE GENETIC COCKTAIL FOR SPECIFICATION OF THE CEREBELLAR ANLAGE

During embryogenesis the CNS suffers several crucial changes. At neural plate stages the brain is an apparent homogenous sheet of epithelial cells, induced during gastrulation by the dorsal lip of the blastopore in amphibians (Spemann and Mangold, 1924), or by the Hensen´s node in amniotes (Figdor and Stern, 1993). During the process of neurulation the neural plate pursues morphological differentiation, its edges thicken and roll up to close dorsally in order to form the neural tube. The anterior portion of the neural tube is undergoing drastic changes generating, by differential proliferation, the three primary brain vesicles: the forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon); the caudal neural tube remains with a cylindrical shape and generates the spinal cord (Martinez and Puelles, 2000). The constriction between the midbrain and hindbrain bulges is called the isthmus. 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

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known as r0 (the isthmus) and r1-r7, followed by the pseudorhombomeres r8-r11 (Fig. 1A; Cambronero and Puelles, 2000). Thus, the cerebellum originates 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 (Fig. 1A; Martinez et al., 2012). Historically, Wilhelm His (1890) proposed already that 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.

In the late 1980s and the 1990s, homotopic and isochronic quail-chick grafting experiments consistently showed that the caudal part of the early midbrain vesicle had a peculiar “morphogenetic activity” and generated the rostral and medial part of the prospective cerebellum (the isthmus; Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990; Alvarez- Otero et al., 1993, Marin and Puelles, 1994). Other specific locations of the developing neural primordium have been identified and in fact, do regulate the identity and regional polarity of neighboring neuroepithelial regions. These local signaling centers are called secondary organizers, (Ruiz i Altaba, 1998; for review see Echevarria et al., 2003). Thus, these secondary organizers operate 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 anterior-posterior (AP) or dorsal-ventral (DV) axis and they regionalize the anterior neural plate and neural tube (Meinhardt, 1983; Figdor and Stern, 1993; Wassef and Joyner, 1997; Rubenstein et al., 1998; Joyner et al., 2000). At the A-P axis, 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 (MHB; Vieira et al., 2010). Thus, the latter is contained at the isthmic constriction (IsO; Figure1B), which has been extensively studied during the last decade (Martinez and Alvarado-Mallart, 1989; for review see Martinez, 2001; Wurst and Bally-Cuif, 2001; Echevarria et al., 2003; Hidalgo-Sanchez et al., 2005; Aroca and Puelles 2005; Partanen 2007; Nakamura et al., 2005). This region is involved in maintaining the MHB 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; Rhinn and Brand, 2001; Martinez 2001; Crespo-Enriquez et al., 2012; Martinez et al., 2013).

As shortly indicated above, the earliest molecular event for the IsO specification is the differential expression in the neural plate of *Otx2* in the rostral neuroepithelium and a *Gbx2* in the posterior domain (Broccoli et al., 1999; Wassarman et al., 1997; Shamim and Mason, 1998; Katahira et al., 2000; Fig. 1B). Both domains become mutually excluded and complemented (Millet et al., 1999; Liu and Joyner, 2001; Garda et al., 2001). The caudal limit of *Otx2* expression and the rostral limit

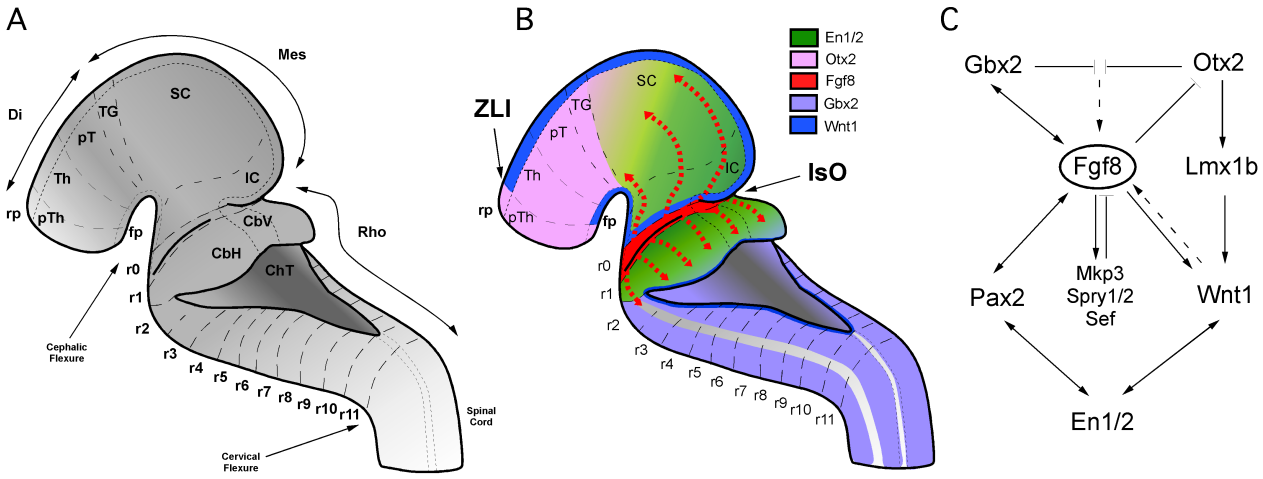

Fig. 1. (A) Representative drawing of a ·3D mouse neural tube of E11.5 viewed caudal wards at which the transversal and longitudinal limits were delineated on the diencephalic (Di), mesencephalic (Mes) and rhombencephalic (Rho) brain subdivisions (see Martinez et al., 2012). **(B)** Shows in a color-coded distribution of the main gene expression patterns at the isthmic region. Red arrows depicted the planar induction direction and propagation of the morphogen FGF8 from the Isthmic organizer (IsO). **(C)** Molecular interaction diagram with the most important genes involved in the patterning and specification of Mes and Rho vesicles.. CbH, cerebellar hemisphere; CbV, cerebellar vermis; ChT, choroid tela; Di, diencephalon; fp, floor plate; IC, inferior colliculus; pT, pretectum; pTh, prethalamus; r0-r8, rhombomeres 0-8; Rp, roof plate; SC, superior colliculus; TG, tegmental grey; Th, thalamus; ZLI, zona limitans intrathalamica.

of *Gbx2* will therefore mark the molecular MHB (Hidalgo Sanchez et al., 1999; Millet et al., 1996; Martinez, 2001).

Some of the key experiments, revealing the molecular nature and regulation of the signals for the specification of the IsO, were performed almost 20 years ago. A member of the fibroblast, growth factor (FGF) family, *Fgf8*, was strongly expressed at the anatomical constriction between the rostral hindbrain and caudal midbrain (Heikinheimo et al., 1994; Crossley and Martin 1995; Fig. 1B). 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; Garda et al., 2001). *Fgf8* expression is first activated at E8.5 in mice at the interface of *Otx2* and *Gbx2* positive neuroepithelial cells. The protein wingless 1 (Wnt1; a signal molecule for proliferation; Danielian and McMahon, 1996) and the homeodomain transcription factors of Engrailed family *En1* and *En2* (Fig. 1B, C) are expressed across the incipient boundary, with a maximum expression level at the *Fgf8* positive domain, showing decreasing gradients oriented either rostrally towards mesencephalic epithelium or caudally towards rhombencephalic epithelium Shortly later, *Wnt1* is 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. Although early *Fgf8* expression appears in the territory coexpressing *Otx2 /Gbx2* (Garda et al., 2001), 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* (Joyner, 1996; Rowitch and McMahon, 1995; Hidalgo Sanchez 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 (Fig. 1C; Vieira et al., 2010). Lately, it is proposed that *Gbx2* and *Fgf8* are sequentially required for formation of the MHB, playing a crucial role in maintaining here a boundary of cell lineage by restricting cell movement (Sunmonu et al., 2011). More recently it is proposed a bi-modal function of Wnt signaling directing the FGF8 activity gradient for regulating neuronal differentiation in the midbrain (Dyer et al., 2014).

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. At the stabilized *Otx2*-*Gbx2* boundary, the *Wnt1*, *En1*, *Fgf8*, and *Pax2* genes initiate cross-regulatory interactions and soon become interdependent, as schematized in Figure1C. Once established, the function of the MHB organizer relies on each member of this genetic network. Thus, alterations of *Fgf8* and *Gbx2* gene expression lead to a massive disruption of the mid-hindbrain neural territory by gene patterning deregulation (Wassarman et al., 1997; Martinez, 2001). 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 (Basson et al., 2008; Chi et al., 2003; Nakamura et al., 2005). On the other hand, in the basal plate, FGF8 gradient is crucial for cell survival and, together with Sonic Hedghog (SHH), essential for the development dopaminergic and serotonergic fates of progenitor cells, (Wurst and Bally-Cuif, 2001; Chi et al., 2003; Puelles and Rubenstein, 2003; Prakash et al., 2006).

Importantly, FGF8 signaling spread 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, a absorptive function of the receiving cells regulated by receptor-mediated endocytosis (Yu et al., 2011; 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* (Echevarria et al., 2005), *Sef* (Furthauer et al., 2002; Tsang et al., 2002) and *Sprouty1/2* (Spry1/2; Minowada et al., 1999; Echevarria et al., 2005b; Fig. 1C). 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 mice 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.

MOLECULAR SPECIFICATION OF THE CEREBELLAR NEURAL SUBPOPULATION

Neurochemically, the cerebellar cortex contains two glutamatergic neuronal subtypes (granule and unipolar brush cells) and six GABAergic subtypes (Purkinje, Golgi, Lugaro, Stellate, Basket and unipolar brush cells). The deep cerebellar nuclei (DCN; Figs. 2, 3A-D), contain both GABAergic interneurons and glutamatergic projection neurons (Carletti and Rossi, 2008; Hoshino, 2006; Leto et al., 2006; Wang and Zoghbi, 2001).

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 rhombic lip (Fig. 3A-D; RL) and ventricular zone (VZ), (Figs. 2, 3; Sotelo, 2004; Millen and Gleeson, 2008). The first germinal center, the external granule layer (EGL), originates from the RL (RL Fig. 2), which locates at the interface of the dorsal neural tube and the extended roof plate of the 4th ventricle (the choroid plexus; Wingate, 2001). The EGL contains early granule cell precursors that produce granule cells. Also, the glutamatergic DCN neurons and unipolar brush cells are derived from the RL (Carletti and Rossi, 2008). Therefore, all glutamatergic neurons in the cerebellum appear to originate from the RL. The rostral RL expresses *Math1* (also called *Atoh1*; Akazawa et al., 1995) 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 choroid plexus (Chp; Basson et al., 2008; Fig. 2). Thereafter and during the first 2 postnatal weeks in mice, granule cell precursors continue differentiating and migrating radially through the molecular cell layer (Fig. 3B; MCL) and through the purkinje cell layer (Fig. 3C; PCL) to form the final internal granular cell layer (Fig. 3D; GCL) leaving their bifurcated axons in the MCL (the parallel fibers; Fig. 3A; Hatten and Heintz, 1995; Wang et al., 2005). The second germinal center, the VZ, has been thought to give rise to cerebellar GABAergic neurons (Carletti and Rossi, 2008; Hoshino, 2006; Altman and Bayer, 1997; Sotelo, 2004; Sudarov and Joyner, 2007; Fig. 2). Genetic fate-map analysis showed that pancreas transcription factor 1 (*Ptf1a*), which encodes a bHLH transcription factor, is expressed at the VZ and required for generating all cerebellar GABAergic neurons including the PCs. (Figs. 2, 3A, 3C; Hoshino et al., 2005; Hoshino, 2006).

Thus, *Math1* and *Ptf1a* participate in regionalizing the cerebellar neuroepithelium, and define two distinct territories, the VZ (*Ptf1a* positive) and the rostral RL (*Math1* positive), which generate GABAergic and glutamatergic neurons, respectively (Hoshino et al., 2005; Pascual et al., 2007; Fig. 2). In addition to GABAergic neurons, progenitor cells located in the ventricular zone of the fourth ventricle also give rise to Bergmann glial cells (BG). During development, the processes of BG provide structural support to the expanding cerebellar plate. Radial Bergmann fibers act as essential guide rails for the migration of granule cells (Rakic, 1990) and contribute to the elaboration of Purkinje cell dendrites (Yamada et al., 2000) and stabilize synaptic connections onto these neurons (Lino et al., 2001).

NEUROPATHOLOGICAL ALTERATIONS REGARDING DEFECTS IN THE ISTHMIC ORGANIZER

The cerebellum, with its stereotyped circuitry, contributes to motor learning and correction of motor acts. Typical symptoms of cerebellar dysfunction include dysergia (problems with measuring appropriate muscle force), dysmetria (improper interpretation of distance), ataxia (disordered movement) and dysdiadochokineasia (inability to perform rapidly alternating movements). 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 (Villanueva, 2012).

Synergy between neuroimaging and molecular genetics has greatly improved our understanding of human developmental disorders arisen from the embryonic midbrain and hindbrain regions (Barkovich et al., 2007, 2009). Thus, several men-

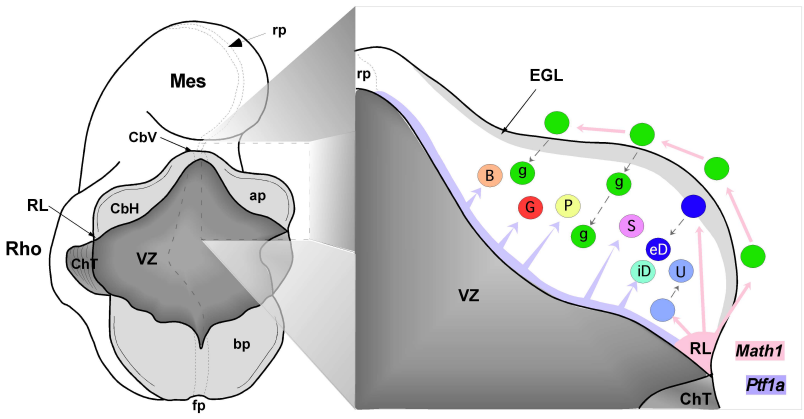

Fig. 2. Schematic representation of a transversal section made at the rhombencephalic anlage leaving the fourth ventricle visible and in which a zoom of the cerebellar anlage is made. In the zoomed region the two main migratory mechanism (radial (blue arrows an lines) and tangential (pink arrows) and the different neuroblast population migratory routes according to their specification site and due to the main two genes involved (*Math1* from the ventricular zone (VZ; glutamatergic source) and *Ptf1a* from the rhombic lip (RL; GABAergic source). ap, alar plate; B, bascket cells; bp, basal plate; CbH, cerebellar hemisphere; CbV, cerebellar vermis; ChT, choroid tela; eD, excitatory deep cerebellar nuclei neurons; EGL, external granular layer; fp, floor plate; g, granular cells; G, Golgi cells; iD, inhibitory deep cerebellar nuclei neurons; Mes, mesencephalon; P, Purkinje cells; Rho, rhombencephalon; rp, roof plate; S, stellate cells; U, unipolar cells.

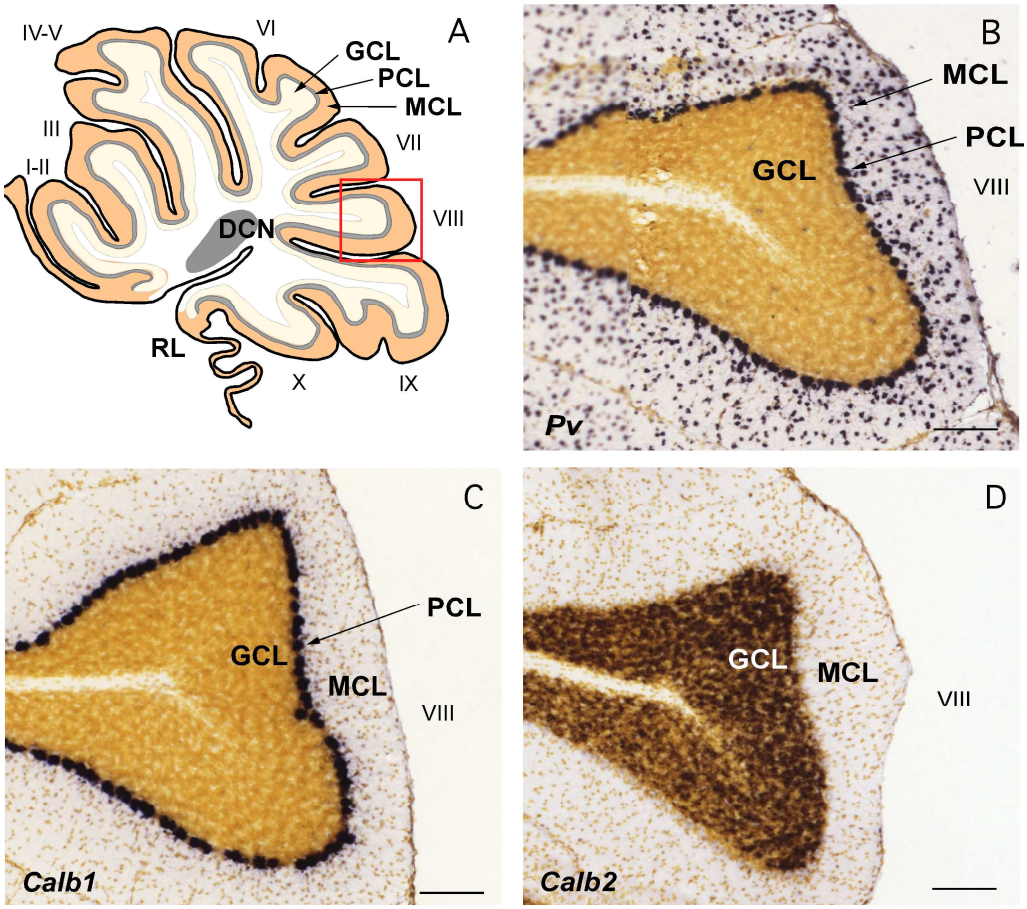

Fig. 3. (A) Representative drawing of an adult cerebellum parasagittal section, showing the different folia distribution from rostral (right) to caudal (left) and the stereotyped layer composition. The red square at folia VIII depicts the photomicrograph taken from Allen brain atlas (http://www.brain-map.org/) p56 mouse brain in which in situ hybridization for parvalbumin (*Pv*) revealing Purkinje cells at the Purkinje cell layer (PCL), Basket and Stellate cells at the molecular cell layer (MCL) **(B)**, Calbindin to reveal Purkinje cells **(C; *Calb1*)** and Calretinin to reveal mostly granule cell population at the granule cell layer (GCL) **(D; *Calb2*).** DCN, deep cerebellar nuclei; I-X, cerebellar folia I-X. Scale bar: 100µm.

tal retardation syndromes or some autism spectral disorders (ASD; Soto-Ares et al., 2003; Courchesne et al., 2005) are commonly related to malformations of the cerebellum. For example, the observation of a human brain through neuroimaging techniques, showing a shortened midbrain and/or elongated pons is normally associated with an enlargement of anterior part of the vermis, and partially missing of mesencephalic tissue. This malformation is presumed to result from a predominance of *GBX2* over *OTX2* transcription factors and consequently a rostral shift of the IsO (Chizhikov and Millen, 2003; Barkovich et al., 2009; Barkovich, 2012). Another example is found in patients lacking of the *MID1* gene. By neuroimaging techniques, brains are observed with a hypoplasia of the anterior cerebellar vermis (Pinson et al., 2004; Fontanella et al., 2008). Using mice lacking *Mid1* gene showed also vermis hypoplasia. In fact, genetic investigations in these mice demonstrated a correlation of the morphology (i.e. rostralization of the isthmus) with a down- regulation of morphogen *Fgf17*, an important early signaling molecule to regulate proliferation and differentiation of midline cerebellar structures (Lancioni et al., 2010; Chi et al., 2003; Xu et al., 2000).

Future research using this combination of techniques will clarify our incomplete knowledge of the clinical consequences of hindbrain and cerebellar anomalies (Doherty et al., 2013). Moreover, 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 better the human brain anomalies.

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