Developmental Biology 367 (2012) 25–39

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

The engrailed homeobox genes are required in multiple cell lineages to coordinate sequential formation of fissures and growth of the cerebellum

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ARTICLE INFO

Article history:
Received 19 December 2011
Received in revised form
30 March 2012
Accepted 11 April 2012
Available online 28 April 2012

Keywords:
En1 and *En2*
Brain development
Foliation
Transcription factors
Patterning
Conditional knockouts
CRE

ABSTRACT

The layered cortex of the cerebellum is folded along the anterior-posterior axis into lobules separated by fissures, allowing the large number of cells needed for advanced cerebellar functions to be packed into a small volume. During development, the cerebellum begins as a smooth ovoid structure with two progenitor zones, the ventricular zone and upper rhombic lip, which give rise to distinct cell types in the mature cerebellum. Initially, the cerebellar primordium is divided into five cardinal lobes, which are subsequently further subdivided by fissures. The cellular processes and genes that regulate the formation of a normal pattern of fissures are poorly understood. The engrailed genes (*En1* and *En2*) are expressed in all cerebellar cell types and are critical for regulating formation of specific fissures. However, the cerebellar cell types that *En1* and *En2* act in to control growth and/or patterning of fissures has not been determined. We conditionally eliminated *En2* or *En1* and *En2* either in both progenitor zones and their descendents or in the two complementary sets of cells derived from each progenitor zone. *En2* was found to be required only transiently in the progenitor zones and their immediate descendents to regulate formation of three fissures and for general growth of the cerebellum. In contrast, *En*1 and *En*2 have overlapping functions in the cells derived from each progenitor zone in regulating formation of additional fissures and for extensive cerebellar growth. Furthermore, *En1/2* function in ventricular zone-derived cells plays a more significant role in determining the timing of initiation and positioning of fissures, whereas in upper rhombic lip-derived cells the genes are more important in regulating cerebellar growth. Our studies reveal the complex manner in which the *En* genes control cerebellar growth and foliation in distinct cell types.

Introduction

Consisting of less than 15% of the total volume in the brain, but containing more than 50% of its neurons, the murine cerebellum plays a key role in coordinating cell communication between the cerebral cortex and body (Llinas, 1975; Manto, 2008). In humans, the cerebellum controls precision and timing of locomotive movements, as well as higher order functions such as language acquisition, attention and emotional responses (Kandel et al., 2000; Manto, 2008). The cerebellum is patterned during development along the three axes. At the level of morphology the medial-lateral axis is subdivided into the vermis, paravermis, hemispheres and flocculi/paraflocculi, based on their distinct patterns of lobules (or folia) separated by fissures along the anterior–posterior axis (Altman and Bayer, 1997). The mammalian vermis can be divided into ten basic lobules (Larsell, 1952; Larsell, 1970) and in the murine vermis there are eight to ten lobules (I–X from anterior to posterior) depending on genetic strain, whereas only four lobules exist in the hemispheres. The production of lobules through the outgrowth of the surface of the cerebellum between fissures in higher organisms results in a great increase cell number, and thus the accommodation of more neural circuits compared to cerebella with a smoother surface. The pattern of the lobules is dependent upon the timing and placement of fissures, and it is therefore critical to study the genetic pathways that regulate formation of fissures in mammals. The generation of a complex structure such as the cerebellum requires precise regulation of cell specification, proliferation, differentiation and migration, and these processes must occur in a particular developmental sequence. Whereas genes required for specification and differentiation of various cell types in the cerebellum have been identified, genes that coordinate all the

Abbreviations: VZ, ventricular zone; RL, rhombic lip; DCN, deep cerebellar nuclei; gcs, granule cells; gcps, granule cell precursors; IGL, internal granule cell layer; EGL, external granule cell layer; pc, preculminate; ppy, prepyramidal; pr, primary; prc, precentral; sec, secondary; po, posterolateral; μm, micrometer

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0012-1606/$ - see front matter © 2012 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.ydbio.2012.04.018

processes to produce the reproducible 3-dimensional structure of the cerebellum are poorly understood.

Development of the cerebellum begins with the specification of the cerebellar anlage from dorsal rhombomere 1 (r1), and in the mouse, this process begins at embryonic day 8.5 (e8.5) (Wingate and Hatten, 1999; Zervas et al., 2004). The cerebellum is unique in the brain in that it has two transcriptionally and spatially discrete progenitor zones: a ventricular zone (VZ) that lines the 4th ventricle, and a structure called the upper rhombic lip (RL) that runs along the posterior edge of the cerebellar anlage. The VZ expresses the pancreas transcription factor 1 alpha gene (*Ptf1a*) and gives rise to gamma-aminobutyric acid (GABAergic) interneurons, Purkinje cells and all glia, whereas the RL expresses *Atoh1* and produces all glutamatergic neurons including those of the deep cerebellar nuclei (DCN) and granule cells (gcs) of the internal granule cell layer (IGL) (Ben-Arie et al., 1997; Hashimoto and Mikoshiba, 2003; Hoshino et al., 2005; Machold and Fishell, 2005; Sudarov et al., 2011). Post-mitotic cells exiting the VZ primarily migrate radially and settle in specific layers of the cerebellar cortex, whereas cells exiting the RL initially migrate along the surface of the developing cerebellum (Altman and Bayer, 1997; Machold and Fishell, 2005). DCN neurons leave the RL first and after reaching the rostral nuclear transitory zone descend ventrally and form three pairs of nuclei along the medial–lateral axis (Altman and Bayer, 1997). The granule cell precursors (gcps) in contrast form a proliferative secondary precursor zone on the surface of the cerebellum called the external granule cell layer (EGL) (Altman and Bayer, 1997). Post mitotic granule cells leave the EGL from e18.5 to postnatal day 16 (P16) and migrate down Bergmann glial fibers (a specialized glial cell) past the Purkinje cell layer to form the IGL. The granule cell axons (parallel fibers) form a progressive layer above the Purkinje cells called the molecular layer, which also houses two major interneuron subtypes (Altman and Bayer, 1997). How the correct number of each cell type is produced in each lobule has yet to be determined.

The process of creating lobules and sublobules through the progression of fissure formation, referred to as foliation, can be divided into two developmental stages (Altman and Bayer, 1997). The first stage of foliation begins at embryonic day 16.5 (e16.5) in the mouse and results in the production of five cardinal lobes in the vermis separated by four cardinal fissures, from anterior to posterior named the preculminate (pc), primary (pr), secondary (sec) and posterolateral (po) fissures (Altman and Bayer, 1997; Mares and Lodin, 1970) (see Fig. 7). None of the four cardinal fissures extend laterally through the entire hemispheres, although the primary fissure forms the anterior surface of the hemispheres. The second stage of foliation begins around birth and continues until the EGL is exhausted of cells (Altman and Bayer, 1997). This stage of development expands and divides the five cardinal lobes into lobules/sublobules as the Purkinje cells mature and spread from a multilayer into a monolayer (Altman and Bayer, 1997). The precentral fissure forms to separate a fused lobule I/II from III, and the prepyramidal fissure separates lobule VII from VIII. The first sign of formation of a fissure is an inward bulging of the inner surface of the EGL that is then followed by an indentation of the outer surface and accompanied by distinct changes in the organization of the surrounding cells, referred to as anchoring centers (Mares and Lodin, 1970; Sudarov and Joyner, 2007). However, the genetic mechanism(s) underlying the timing of initiation and positioning of fissures remains poorly understood.

The mouse engrailed1 (*En1*) and engrailed2 (*En2*) genes (Joyner et al., 1985; Joyner and Martin, 1987) encoding homeodomain transcriptional repressors are known to regulate many aspects of cerebellar patterning, including foliation (Cheng et al., 2010; Joyner et al., 1991; Millen et al., 1994; Sgaier et al., 2007) striped gene expression (Sillitoe et al., 2008) and afferent circuit topography (Sillitoe et al., 2010). In the mouse, *En1* expression begins at ~e8.0 and *En2* shortly thereafter in the mesencephalon (midbrain precursor) and r1 (Davis and Joyner, 1988; Davis et al., 1988). *En1/2* continue to be expressed throughout cerebellar development, but become spatially and temporally restricted to defined regions in the cerebellar anlage (Millen et al., 1995; Sgaier et al., 2007; Wilson et al., 2011). By e17.5, parasagittal groups of Purkinje cells express *En1/2* and granule cell precursors express *En1* primarily in the presumptive vermis and *En2* more broadly (Millen et al., 1995; Wilson et al., 2011). During postnatal development and in the adult, *En1* or *En2* continue to be expressed, but by P21 expression is restricted to subsets of Purkinje and DCN cells or granule cells, respectively, as well as interneurons (Wilson et al., 2011). While *En1/2* are known to have dynamic expression patterns throughout cerebellar development, the relationship between the expression of these genes in particular cell types and their roles in regulating foliation remains to be elucidated.

Several studies of null and conditional *En1/2* mutants have defined overlapping and distinct requirements for *En1* and *En2* in cerebellar development. The earliest expression of *En1* is required for specification of the cerebellar anlage, as the absence of *En1* results in loss of the cerebellum by e9.5, unless the mutation is on a C57bl/6 background (Bilovocky et al., 2003; Wurst et al., 1994). In contrast, later expression of *En1* is not required for cerebellar foliation, as one third of outbred conditional mutants ($En1^{Cre/fx}$) that lack *En1* after e9, have normal cerebellar foliation (Sgaier et al., 2007). Unlike *En1*, *En2* is required after e12.5 for cerebellar foliation (Cheng et al., 2010; Joyner et al., 1991; Kuemerle et al., 1997; Millen et al., 1994). The vermal patterning defect in *En2* null mutants, a posterior shift of lobule VIII, results from a delay in formation of the secondary fissure and premature initiation of the prepyramidal fissure (Millen et al., 1994; Sudarov and Joyner, 2007). In the hemispheres, three rather than four lobules form due to lack of formation of the ansoparamedian fissure, resulting in the amalgamation of the CrusII and Paramedian lobules (Millen et al., 1994). Within their DNA binding domains, EN1 and EN2 share high homology and studies have demonstrated overlapping functions for the two proteins. For example, if the *En2* coding sequence is used to replace that of *En1* ($En1^{2ki/2ki}$ knock-in mice) the *En*1 null phenotype is rescued (Hanks et al., 1995), whereas if *En1* in addition to *En2* is conditionally ablated after e14.5 ($R26^{CreER/+}$; $En1^{fx/-}$; $En2^{fx/-}$ mice given tamoxifen at e13.5 and e14.5) the size of the cerebellum is further reduced and additional lobules in the vermis are disrupted (Cheng et al., 2010; Sgaier et al., 2007). The *En2* null mutant hemisphere foliation phenotype is only reproduced, however, if *En2* (or both *En* genes) are inactivated by e11. While these studies revealed a role for *En1/2* in regulating foliation and growth after e14.5, the cells in which the *En* genes function to regulate cerebellar development were not defined.

As a means to understand how the *En* genes regulate foliation and growth of the cerebellum, we inactivated *En2* alone or *En1* and *En2* in cells of the two progenitor zones and/or their descendents. Inactivation of *En1/2* in both progenitor zones at ~e10.5 resulted in a profound inhibition of expansion of the vermis. In contrast, deletion of the *En* genes in the cells derived from the two progenitor zones revealed that both *En1* and *En2* are required within these cells for foliation and general growth. Moreover, combined EN1/2 function is required independently in VZ- and RL-derived cells to regulate foliation, with VZ-derived cells playing a greater role. In addition, RL-derived cells play a prominent role in cerebellar growth. Finally, developmental studies uncovered distinct alterations in the timing of formation of particular fissures depending on the cell types in which the *En* genes are intact. Thus, *En1* and *En2* act together in multiple cerebellar cell types to determine overall growth and formation of particular fissures. In this way the *En* genes are critical for determining the number of cells allocated