

A role for *En-2* and other murine homologues of *Drosophila* segment polarity genes in regulating positional information in the developing cerebellum

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SUMMARY

To gain insight into the molecular genetic basis of cerebellar patterning, the expression patterns of many vertebrate homologues of *Drosophila* segment polarity genes were examined during normal and abnormal cerebellar development, including members of the *En*, *Wnt*, *Pax*, *Gli* and *Dvl* gene families. Five of these genes were found to show transient, spatially restricted patterns of expression. Strikingly, expression of *En-2*, *En-1*, *Wnt-7B* and *Pax-2* defined eleven similar sagittal domains at 17.5 dpc, reminiscent of the transient sagittal domains of expression of Purkinje cell markers which have been implicated in cerebellar afferent patterning. Postnatally, transient anterior/posterior differences in expression were observed for *En-2*, *En-1*, *Gli* and *Wnt-7B* dividing the cerebellum into anterior and posterior regions. The expression patterns of these genes were altered in cerebella of *En-2* homozygous mutant mice,

which show a cerebellar foliation patterning defect. Strikingly, four of the *Wnt-7B* expression domains that are adjacent to the *En-2* domains are lost in *En-2* mutant embryonic cerebella. These studies provide the first evidence of a potential network of regulatory genes that establish spatial cues in the developing cerebellum by dividing it into a grid of positional information required for patterning foliation and afferents. Taken together with previous gene expression studies, our data suggests that eleven sagittal domains and at least two anterior/posterior compartments are the basic elements of spatial information in the cerebellum.

Key words: cerebellum, patterning, vertebrate segment polarity genes, *Engrailed*, gene expression, in situ hybridization

INTRODUCTION

The cerebellum offers a unique system to study CNS patterning. It is composed of only a few cell types, each arranged in a highly organized laminar structure with a regular set of folds and a well defined and reiterated neural circuitry. Patterning of the cerebellum can be divided into several distinct processes. The first involves early regionalization of the neural tube, resulting in the formation of a cerebellar territory. Histogenesis of specific neural cell types then proceeds in a temporally and spatially defined manner. Each cell type then undergoes an elaborate differentiation process that includes cell migration, axon and dendrite growth and targeting, and changes in cell body shape. Granule cells also undergo extensive proliferation. These events are co-ordinated during cerebellar development to generate the final folded morphology, laminar structure and neural network of the adult cerebellum.

Although often noted for its uniform structure, functional anatomical and biochemical studies have demonstrated that the cerebellum is highly patterned. Most obvious are the fissures which divide the cerebellar cortex into parallel folia in the medial to lateral axis. These fissures are highly conserved

within and across species and are thought to arise by differential proliferation of the external granule layer (EGL) (Mares et al., 1970; Mares and Lodin, 1970). The cerebellum is also subdivided into parallel sagittal domains based on gene expression and afferent projections. A number of proteins and glycoproteins have been described that are transiently expressed from 15.5 days post coitus (dpc) to postnatal day 5 (P5) in subsets of differentiating Purkinje cells arranged in reproducible sagittal arrays (reviewed in Wassef et al., 1992a). These include calbindin, c-GMP-dependent protein kinase, Purkinje cell-specific glycoprotein, PEP 19 and L7/pcp-2 and are distinct from the zebrins which also are expressed in sagittal subsets of Purkinje cells but not until P14 (reviewed in Hawkes, 1992). Both spinocerebellar and olivocerebellar afferent terminals are also arranged in sagittal columnar patterns throughout the cerebellar cortex (Ito, 1984; Gravel et al., 1987 and Gravel and Hawkes, 1990). It has been proposed that sagittal subsets of Purkinje cells are individually 'labelled' by a combination of separate Purkinje cell antigens and that these labels encode a topographic map of the cerebellar cortex for guiding incoming afferents (Wassef et al., 1992a). Recent studies indicate that the sagittal expression of at least L7/pcp-

2 is intrinsic to Purkinje cells and independent of afferent input and that positional information must be encoded in Purkinje cells in the early cerebellar anlage (Oberdick et al., 1993). Thus, although all cerebellar neurons of a particular neuronal type undergo the same general differentiation program, there must be an underlying scaffold of positional information which regulates the global patterning of folial and afferent targeting.

An understanding of the genetic regulation of positional information in the developing cerebellar anlage has remained elusive. Phenotypically, cerebellar foliation has been easier to assess than afferent patterning. Although many cerebellar mutants display very abnormal foliation, in all but *Engrailed-2* (*En-2*) mutants, this is a secondary effect of disrupted laminar formation. Two pieces of evidence suggest that the genetic control of cerebellar foliation is separable from the genetic control of neuronal histogenesis, differentiation and cytoarchitecture formation. Subtle differences in the positioning of small sulci are found in different inbred strains of mice (Inouye and Oda, 1980) in the absence of laminar defects. Mapping studies have identified two loci responsible for these small foliation effects; *cerebellar folial pattern-1* (*Cfp-1*) and *declival sulcus of cerebellum* (*dsc*) (Neumann et al., 1990; Cooper et al., 1991), but neither locus has been characterized at a molecular level. Stronger evidence comes from mice homozygous for targeted mutations in the *En-2* gene (Joyner et al., 1991; Millen et al., 1994). These mice show distinct foliation patterning alterations in major fissures of both the vermis and the hemispheres of the cerebellum as well as a 30% reduction in cerebellar size. The cerebellar cytoarchitecture in *En-2* mutants, however, appears normal.

The cerebellum of *En-2* mutants is reduced in size from at least 15.5 dpc and the foliation patterning defect becomes evident during postnatal development, affecting the positioning of three posterior fissures. In the vermis of *En-2* mutants, there is abnormal positioning and/or depth of two fissures and in the hemispheres, one fissure is absent. The anterior fissures appear to be unaffected. Furthermore, in the adult, changes in regional Purkinje and granule cell gene expression in the posterior of the cerebellum have been observed, possibly reflecting changes in lobule identity (Millen et al., 1994).

The two mouse *En* genes (*En-1* and *En-2*) were isolated based on their homology to the *Drosophila* segment polarity gene, *engrailed* (Joyner et al., 1985; Joyner and Martin, 1987). From 8.5 dpc, both genes are expressed in a broad band across the developing mid/hindbrain junction, a region from which the cerebellum is derived (Davis and Joyner, 1988; Davis et al., 1988, 1991; Davidson et al., 1988). In the adult, expression of both genes is limited to specific neuronal groups in the pons region and substantia nigra. In the cerebellum, *En-1* is not expressed, whereas *En-2* is expressed in cells of the granule and molecular layers. In contrast to the *En-2* phenotype, mice homozygous for a targeted mutation at the *En-1* locus die at birth and lack a large portion of the cerebellum and colliculi (Wurst et al., 1994). The differences in phenotype severity have been shown to be due to functional overlap between the two genes (Hanks et al., 1995).

To further explore the role of *En-2* in cerebellar patterning, we analyzed *En-2* gene expression during cerebellar development to investigate at which stages *En-2* could be involved in cerebellar patterning. *En-1* expression was also analyzed to determine whether it could have an additional or overlapping

role during cerebellar development. We observed that *En* gene expression was complex and dynamic, with changing spatially restricted expression patterns between 15.5 dpc and postnatal day 8 (P8). To identify other candidate genes controlling cerebellar patterning, expression analysis was conducted focusing on other vertebrate homologues of *Drosophila* segment polarity genes, since these genes are part of a genetic network involving *engrailed* during pattern formation in the fly (recently reviewed by Perrimon, 1994). The analysis included members of the *Wnt*, *Pax*, *Gli* and *Dvl* gene families. Several members of these gene families were found to show spatially restricted expression patterns in sagittal domains in the late embryonic cerebellar anlage and anterior/posterior domains in the early postnatal cerebellum. Furthermore, specific elements of the expression patterns were altered in *En-2* homozygous mutant cerebella. Based on the expression patterns of these genes and the *En-2* mutant phenotype, we propose that members of a genetic network including vertebrate homologues of the *Drosophila* segment polarity genes divide the embryonic cerebellum into eleven sagittal domains and at least two anterior/posterior domains. These domains of gene expression form part of the basic system of positional information regulating the patterning of cerebellar foliation and afferent targeting.

MATERIALS AND METHODS

Mouse strains and tissue preparation

Mice carrying the *En-2^{hd}* mutation were maintained on a 129/Sv inbred background. Offspring were genotyped as described by Joyner et al. (1991). Littermate 129/Sv wild-type animals were used as controls. For the studies of wild-type RNA in situ analysis and for analysis by immunohistochemistry, CD1 outbred animals were used where stated.

For immunohistochemistry, brains were dissected at various stages and frozen directly into OCT (Tissue-Tek). For in situ analysis of brains at late embryonic stages, the brains were dissected directly into 4% paraformaldehyde in PBS and fixed overnight at room temperature (RT), then transferred to 70% ethanol in saline at RT for a period ranging from several hours to overnight. Alternatively, intact heads of late embryonic stage mice were fixed in 4% paraformaldehyde in PBS overnight at RT, transferred to 70% ethanol in saline at RT for several hours to overnight and then the brains were dissected directly into 70% ethanol. For analysis of postnatal stages, the brain tissue was dissected (without perfusion) directly into 4% paraformaldehyde in PBS, fixed for a minimum of 24 hours at RT, then transferred to 70% ethanol for at least 12 hours. At all stages, for both immunohistochemistry and in situ analysis, between 10–25 individual brains were examined for expression of each gene to ensure that patterns of gene expression were consistent and reproducible.

Immunohistochemistry

Air-dried 10 µm cryostat sections of fresh, frozen brain tissue were rehydrated in PBS, postfixed for 15 minutes in 2% paraformaldehyde in PBS, then blocked in PBS, 2% milk powder and 0.3% Triton X-100 (PBSMT), two times for 5 minutes, followed by a 2 hour RT incubation with a 1:1000 dilution of a-Enhb-1 crude serum (Davis and Joyner, 1988) in PBSMT. Slides were washed two times for 5 minutes in PBS, 0.3% Triton X-100 and 2% BSA (PBT) and blocked as above. This was followed by a 2 hour RT incubation with goat-anti-rabbit IgG antiserum conjugated with horseradish peroxidase (Pierce ImmunoTechnology), diluted 1:500 in PBSMT. Slides were washed as above, rinsed in PBT and incubated for 10 minutes with 0.03%

diaminobenzidine and 0.5% NiCl₂ in PBT. H₂O₂ was added to 0.03%, the reaction was monitored and the staining was stopped after approximately 10 minutes. Slides were dehydrated through ethanol, cleared in xylene and mounted.

RNA in situ hybridization

RNA in situ analysis was conducted as described by Davis et al. (1988); Hui et al. (1994). Near adjacent or adjacent sections were used in most cases to allow a more accurate comparison of expression patterns. Both ³⁵S- and ³³P-labelled RNA probes were used. ³³P-labelled probes were observed to significantly lower the background signal, especially on postnatal brain tissue. The *En-2* probe used, was a 800 bp *Bgl*II-*Xba*I, 3' untranslated fragment. The *En-1* probe was a 600 bp *Eco*RI fragment of the 3' untranslated end of the cDNA (Joyner and Martin, 1987). The *Wnt-1*, *Wnt-4*, *Wnt-5A*, *Wnt-6* and *Wnt-7B* probes (Parr et al., 1993) were provided by A. McMahon. Probes for *Wnt-3* (Salinas et al., 1994) and *Wnt-3A* (Roelink and Nusse, 1991) were provided by R. Nusse. *Pax-2* (Dressler et al., 1990), *Pax-3* (Goulding et al., 1991), *Pax-5* (Asano and Gruss, 1992), *Pax-6* (Walther and Gruss, 1991), *Pax-7* (Jostes et al., 1991) and *Pax-8* (Salinas et al., 1994) were provided by P. Gruss. The *Dvl-1* probe (Sussmann et al., 1994) was provided by D. Sussmann. The *Gli-2* and *Gli-3* probes have been previously described (Hui et al., 1994).

RNAse protection assay

Total RNA was extracted using the method of Sekiguchi et al. (1992) from postnatal day 5 (P5) cerebella, dissected from littermates of *En-2*^{hd/+} × *En-2*^{hd/+} crosses. β -actin probe was provided by Janet Rossant. Both the *Pax-3* and *Pax-6* probes were the same as used for in situ hybridization. The assay was conducted as described by Ausubel et al. (1987), using 10 μ g of total cerebellar RNA and 5 × 10⁵ cpm of probe RNA in each reaction. Quantitation was performed on a Molecular Dynamics Image-Quant system.

RESULTS

Spatially restricted expression in the wild-type embryonic cerebellum

In order to examine *Engrailed* expression during embryonic cerebellar development, horizontal and sagittal sections of wild-type cerebella of 15.5 and 17.5 dpc mice were analyzed by both RNA in situ hybridization and immunohistochemistry. This analysis revealed complex and dynamic patterns of *En-2* and *En-1* expression in the developing cerebellum. Fig. 1 shows a summary of the observed expression patterns at 17.5 dpc. At this stage, there were five clear sagittal *En-2* bands of higher expression across the cerebellum in the medial to dorsal horizontal planes of the cerebellum. We define

bands as patches of gene expression in sagittally restricted domains. One single band was located at the midline with two bilaterally symmetrical bands also evident (Fig. 1E,F). Ventrally, high levels of *En-2* expression were limited to the midline (Fig. 1D). *En-1* was restricted to a single midline band, primarily within the medial horizontal plane (Fig. 1H). Both *En-1* and *En-2* high level expression appeared to involve all cell types present in the cerebellum. Analysis using the α -Enhb-1 antiserum, which detects both *En* proteins (Davis et al., 1991), showed that the antibody staining was a composite of both expression patterns indicating that translation is coupled with transcription (data not shown). Spatially restricted expression was evident as early as 15.5 dpc (data not shown).

The sagittal bands of *En* expression at 17.5 dpc were reminiscent of the transient sagittal bands described for expression of early Purkinje cell markers such as *L7/pcp-2*. This suggested that *En-2* may be a critical transcription factor regulating positional information in the cerebellum. In an attempt to identify other potential regulatory genes, an expression analysis of vertebrate homologues of *Drosophila* segment polarity genes was conducted at 17.5 dpc. Table 1 summarizes the 19 genes used in this study. Most members of the *En*, *Wnt*, *Pax*, *Gli* and *Dvl* gene families were examined. In *Drosophila*, the related genes contribute to a genetic network important for many patterning events during *Drosophila* development (Hooper and Scott, 1992; Perrimon, 1994).

The wild-type expression patterns of all the genes showing spatially restricted expression at 17.5 dpc are presented in Fig. 2 and summarized in Table 1. *Pax-2* and *Wnt-7B* were

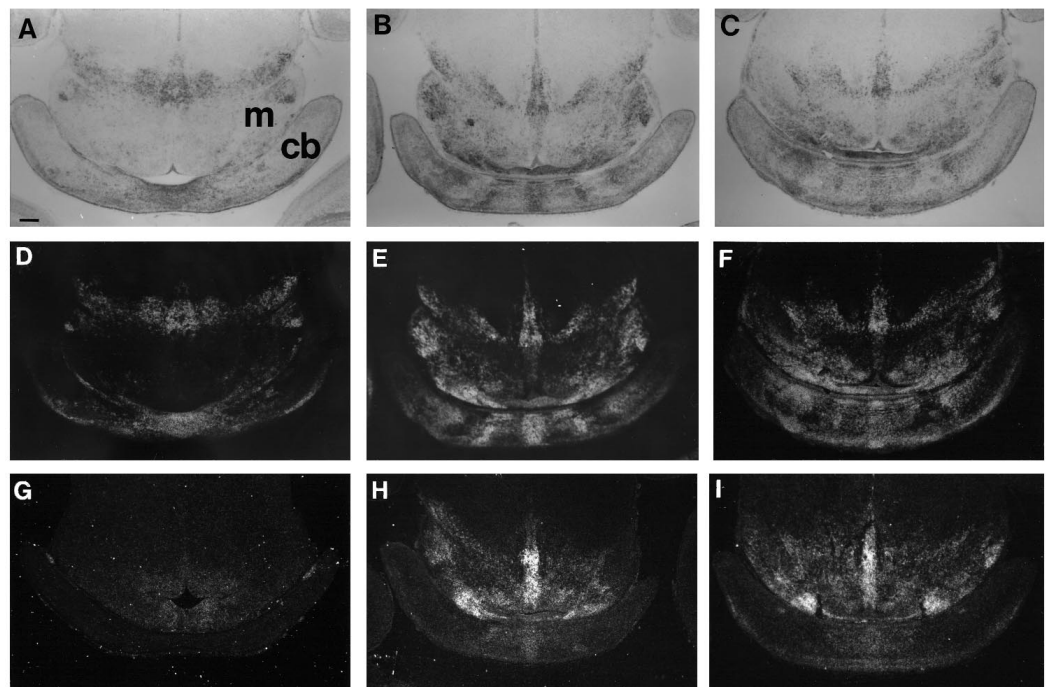


Fig. 1. *Engrailed* expression in the embryonic cerebellar anlage. Bright-field (A-C) and dark-field micrographs (D-I) of ventral (A,D,G), medial (B,E,H) and dorsal (C,F,I) horizontal sections through 17.5 dpc cerebella. Adjacent sections were probed with *En-2*- (D,E,F) or *En-1*-specific (G,H,I) probes. High levels of *En-2* expression were seen ventral/posteriorly. In both medial and dorsal sections, *En-2* was expressed at low levels across the cerebellum, with higher bands of expression superimposed. *En-1* expression was limited to the central regions of medial and dorsal planes. cb, cerebellum; m, midbrain. Anterior is to the top. Scale bars, 100 μ m.

expressed in a complementary pattern to the *En-2* bands (Fig 2G,J), with three bilaterally symmetrical bands corresponding to the *En-2*-negative bands. Although it was difficult to compare the exact boundaries of expression between genes on adjacent sections, the expression patterns of all the genes seemed to identify eleven domains across the cerebellum. We have defined the central band as 'A' and the other bilaterally symmetrical bands as bands 'B-F' (see Fig. 3). Using such nomenclature, the *En-2*-positive bands are bands A,C and E, while the *En-2*-negative (*Pax-2*/*Wnt-7B* positive) bands are B, D and F. Within the bands there was also evidence of spatial restrictions along the anterior to posterior axis when the detailed expression within each band was considered. For example, *Wnt-7B* expression in the B bands was primarily restricted to the posterior half of the band (Figs 2J, 3).

Not all genes expressed in the cerebellum at 17.5 dpc showed spatially restricted expression patterns. *Dvl-1* was highly expressed in all cell types (Fig. 4B), while diffuse expression throughout the cerebellum was observed for *Wnt-3*, *Gli* and *Pax-3* (Fig. 4C-E). Importantly, this indicated that the spatially restricted patterns observed for the other genes did not simply reflect intrinsic differences in cell density across the cerebellum.

Patterns of gene expression are altered in the *En-2* mutant embryonic cerebellum

If the identified genes showing spatially restricted expression patterns are involved in regulating cerebellar patterning, we would expect their expression to be altered in cerebellar patterning mutants. Expression of these genes was therefore analyzed in *En-2* mutants, which show altered folial patterning. To directly compare expression patterns, mutant and wild-type cerebella from heterozygous intercrosses were analyzed at 17.5 dpc (Fig. 2). In addition,

since at 17.5 dpc the *En-2* mutant displays a slight delay in embryonic cerebellar development (Millen et al., 1994), mutant brains were also collected at 17.75 dpc, 5-6 hours later, when the mutant cerebellar primordia had completely fused on the mid-line.

All genes expressed in the wild-type cerebellum were also found to be expressed in the mutant cerebellum. Genes that were not patterned in the wild-type did not become patterned in the mutant (Fig. 4F and data not shown) and genes that were patterned in the wild-type remained patterned in the mutant (Fig. 2). However, the patterned gene expression was altered in *En-2* mutants at both 17.5 and 17.75 dpc. Comparable mutant and wild-type horizontal sections were examined, using fore-, mid- and hind-brain landmarks as guides to avoid differences due to the plane of section. Furthermore, at no plane in the mutant cerebellum were the patterns identical to the

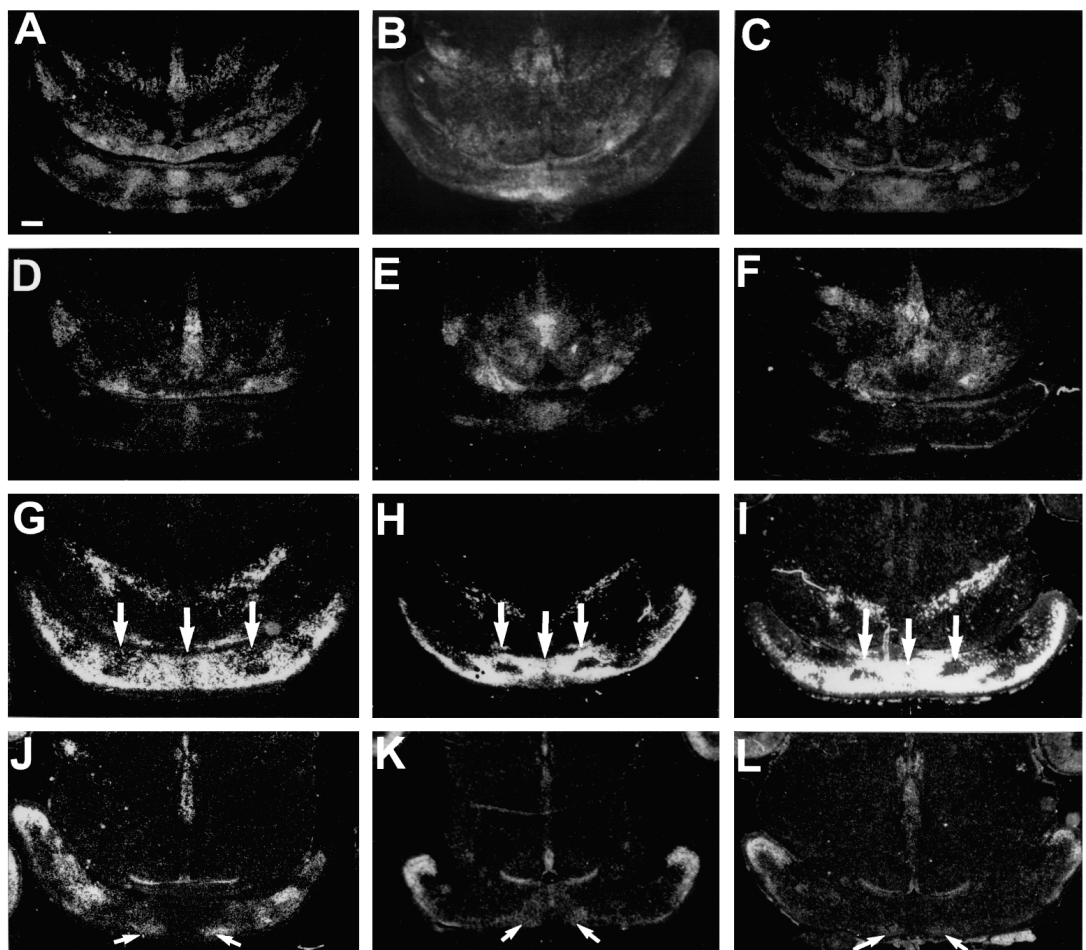


Fig. 2. Spatially restricted gene expression in embryonic wild-type and *En-2^{hd}* homozygous mutant cerebella. In situ hybridization of adjacent or near adjacent sections through the medial horizontal plane of 17.5 dpc wild-type (A,D,G,J), 17.5 dpc (B,E,H,K) and 17.75 dpc (C,F,I,L) *En-2^{hd}* homozygous mutant cerebella. Sections were hybridized with probes specific for *En-2* (A-C), *En-1* (D-F), *Pax-2* (G-I) and *Wnt-7B* (J-L). *En-2* expression was detected at low levels across the wild-type cerebellum (A), with bands of higher expression. At both stages in the mutant, the *En-2* probe detected a broad band of expression centrally (B,C). Although low levels of central *En-1* expression were detected in the wild-type cerebellum (D), the band was broader in the 17.5 dpc mutant cerebellum and no longer detectable by 17.75 dpc (E,F). Wild-type *Pax-2* expression (G) demarkates domains that are complementary to the wild-type *En-2* bands of expression (arrows). The domains of lower expression are closer together in the mutant (H,I). *Wnt-7B* expression also marks similar complementary bands (J). Arrows indicate expression is reduced centrally in the mutant (K,L). Scale bars, 100 μ m.

Table 1. Summary of the expression of vertebrate homologues of *Drosophila* segment-polarity genes in the developing wild-type cerebellum

Genes screened					
Vertebrate genes	<u><i>En-1</i></u>	<u><i>Wnt-1</i></u>	<u><i>Pax-2</i></u>	<u><i>Gli</i></u>	<u><i>Dvl-1</i></u>
	<u><i>En-2</i></u>	<u><i>Wnt-3</i></u>	<u><i>Pax-3</i></u>	<u><i>Gli-2</i></u>	
		<i>Wnt-3A</i>	<i>Pax-5</i>	<u><i>Gli-3</i></u>	
		<i>Wnt-4</i>	<u><i>Pax-6</i></u>		
		<u><i>Wnt-5A</i></u>	<i>Pax-7</i>		
		<i>Wnt-6</i>	<i>Pax-8</i>		
<i>Drosophila</i> homologue	<i>en</i>	<i>wg</i>	<i>prd/gsb</i>	<i>ci</i>	<i>dvl</i>
Genes expressed at 17.5 dpc					
Spatially patterned	<i>En-1</i>	<i>Wnt-7B</i>	<i>Pax-2</i>		
	<i>En-2</i>				
Not patterned		<i>Wnt-3</i>	<i>Pax-3</i>	<i>Gli</i>	<i>Dvl-1</i>
			<i>Pax-6</i> ¹		
Genes expressed postnatally					
Spatially patterned	<i>En-1</i>	<i>Wnt-7B</i>		<i>Gli</i>	
	<i>En-2</i>				
Not patterned		<i>Wnt-3</i>	<i>Pax-2</i>	<i>Gli</i> -2 ²	<i>Dvl-1</i>
		<i>Wnt-3A</i>	<i>Pax-3</i>	<i>Gli</i> -3 ²	
		<i>Wnt-5A</i> ²	<i>Pax-6</i>		

The genes used in the expression analysis are listed in the first part of the table by gene family. The *Drosophila* homologue of each gene family is also listed. Of the genes screened, those found to be expressed in the developing cerebellum are underlined. ¹weakly patterned, ²genes not expressed at P2, but expressed from P5 onwards

wild-type patterns. The medial sections shown in Fig. 2 represent the most patterned sections of the mutant cerebella. A schematic diagram of gene expression in the mutant is shown in a Fig. 3.

We had found previously that transcripts from the targeted *En-2* locus were expressed in the same tissues as the wild-type *En-2* allele (Joyner et al., 1991). To confirm that the spatial pattern of expression of the mutant *En-2* allele was expressed normally, sections from *En-2* heterozygous animals were hybridized with the *En-2* in situ probe to detect both the wild-type and mutant alleles. The combined pattern of expression from the two alleles was found to be indistinguishable from the normal pattern (data not shown). The endogenous *En-2* regulatory elements therefore appear to direct expression from the mutant allele in an *En-2*-like manner. Thus, the probe can be used to detect *En-2* expression in *En-2* mutants. At both stages, *En-2* homozygous mutant cerebella expressed the mutant *En-2* transcript in a broadened mid-sagittal band, with at least, one partial bilaterally symmetrical lateral band (Fig. 2B,C).

Other genes also showed altered expression patterns around the midline of the mutant cerebella (Figs 2, 3). *En-1* expression was decreased and broadened at the midline (Fig. 2E,F). For *Pax-2*, there was a change in the size and spacing of the bands of both positive and negative expression, although the basic pattern persisted (Fig. 2H,I). The widths of the *Pax-2*-expressing B and D bands were reduced in size, resulting in the *Pax-2* non-expressing C bands being closer to the midline. The lateral F band was also slightly enlarged (Fig. 3). We do not believe that the size reduction was due to different planes of section, since at no plane was the spacing similar to wild-type. For *Wnt-7B*, a striking loss of the B and D expressing bands was apparent, whereas the F band of *Wnt-7B* expression was unaffected (Figs 2K,L, 3).

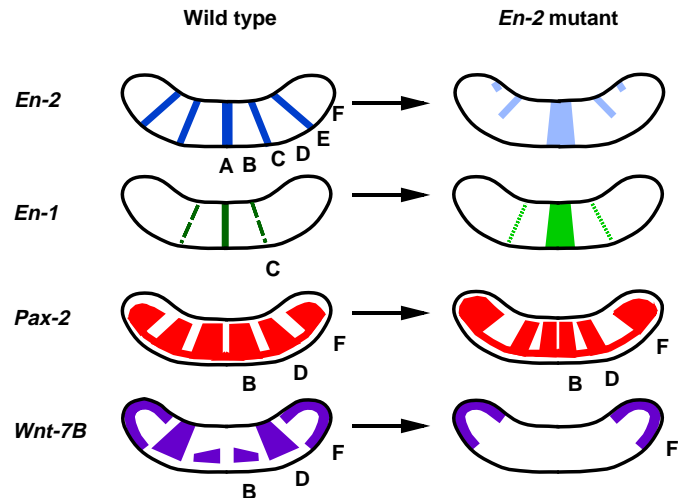


Fig. 3. A schematic representation of gene expression in the wild-type and *En-2*^{hd} mutant cerebellum at 17.5 dpc. Expression patterns of *En-2*, *En-1*, *Pax-2* and *Wnt-7B* are illustrated in schematic medial horizontal sections for both wild-type and mutant cerebella. In wild-type cerebella, eleven sagittal domains are defined as a midline band A and five bilaterally symmetrical bands B-F. The patterns are altered in the *En-2*^{hd} mutant cerebellum as indicated.

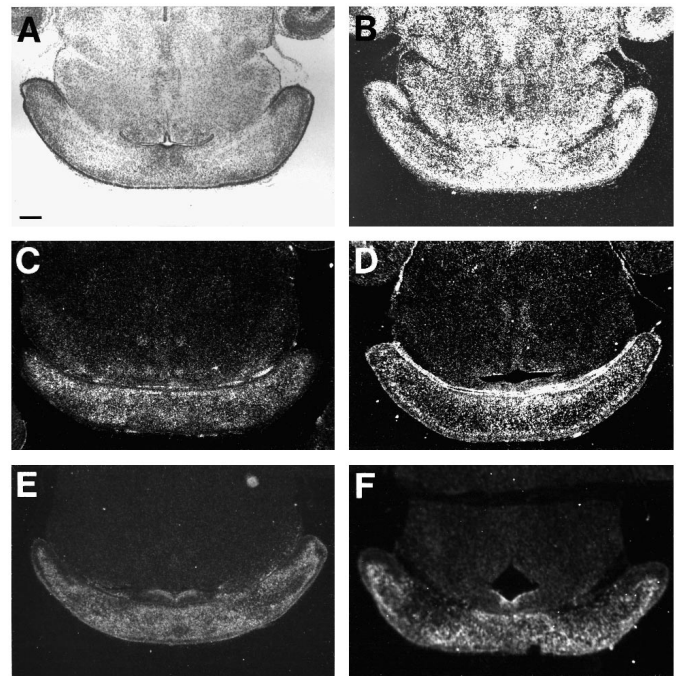


Fig. 4. Not all gene expression is spatially restricted in the developing cerebellum at 17.5 dpc. Bright-field (A) and dark-field (B-F) micrographs of adjacent medial horizontal sections of wild-type 17.5 dpc cerebella probed with *Dvl-1* (B) *Wnt-3* (C) *Gli* (D) and *Pax-3* (E) specific probes. *Pax-3* expression did not change in the *En-2*^{hd} homozygous mutant 17.5 dpc cerebellum (F). Anterior is to the top. Scale bar, 100 μm.

Spatially restricted expression during wild-type postnatal cerebellar development

The adult cerebellar expression patterns that have been reported for some of the genes used in this study (Davis and

Joyner, 1988; Davis et al., 1988; Salinas et al., 1994; Stoykova and Gruss, 1994) are cell-type specific with no evidence of spatially restricted expression. It was therefore of interest to examine the transition between the spatially restricted expression patterns observed in this study and the previously reported, non-patterned, cell-type-specific expression in the adult cerebellum. We chose to examine the postnatal stages of P0, P2, P5 and P8, when the major events of cerebellar foliation and axon targeting are occurring.

In the adult, *En-1* is not expressed in the cerebellum and *En-2* is expressed in the granule cells and cells in the molecular layer (Davis and Joyner, 1988; Davis et al., 1988). Analysis of both *En-1* and *En-2* expression during early postnatal cerebellar development is summarized in Figs 5-8. Postnatally, the expression patterns of both genes changed dramatically. At P0, both medially and ventrally (Fig. 5D-F), *En-2* expression was restricted to a broad band of expression around the mid-line vermis, with little to no expression in the developing hemispheres. Faint, residual mid-sagittal bands of *En-2* were observed only very dorsally (Fig. 5F). As cerebellar development proceeded, expression of *En-2* became strongest in both the EGL and the internal granule layer (IGL), with expression radiating from the mid-line laterally with time. At P2, expression was detected only in the vermis (Fig. 5D-F), whereas at P5, it also included part of the hemispheres (Fig. 5K and data not shown). Along the anterior/posterior axis, in mid-sagittal sections at P2, *En-2* was most strongly expressed in the anterior IGL of the cerebellum to lobule V and in the most posterior lobule X (Fig. 8I). Mid-sagittal sections at P5 revealed that *En-2* was expressed in all lobules in a gradient, with transcripts more prevalent in posterior EGL and IGL cells of the cerebellum (Fig. 8K).

At birth, *En-1* was not detected in the developing cerebellum (Fig. 5G-I), however at P2, faint *En-1* expression was detected on the mid-line (data not shown) and by P5, *En-1* expression was strongest in the internal granule layer of the vermis (Fig. 5L). By P8, no medial to lateral differences were observed for either *En-2* or *En-1*. No *En-1* transcripts were detected in the developing cerebellum

at P21 (data not shown). At P2 and P5, *En-1* expression was restricted to the anterior lobules of the cerebellum, with a boundary of expression at the base of the VIth lobule (Fig. 8M,O). This boundary was consistent at both stages. Similar to *En-2*, no differences in spatial expression were observed at P8 (data not shown).

Postnatally, staining with the α -Enhb-1 antiserum showed that En expression in the EGL was much stronger in the inner migrating cells than the proliferating EGL cells (Fig. 6). In addition, antibody staining did not detect En protein in Purkinje cells beyond P0.

Similar to *En* expression, the other genes examined postnatally had patterns of expression very different from those observed at 17.5 dpc (summarized in Table 1 and Fig. 8). All genes expressed at 17.5 dpc were also expressed postnatally. In addition, *Wnt-5A*, *Gli-2* and *Gli-3*, while not expressed at 17.5 dpc, P0 or P2, showed expression at P5 and P8. Of the six genes showing spatially restricted expression at 17.5 dpc, only *En-1*, *En-2* and *Wnt-7B* had obvious spatial patterning at P2. Additionally, *Gli*, a non-patterned gene at 17.5 dpc,

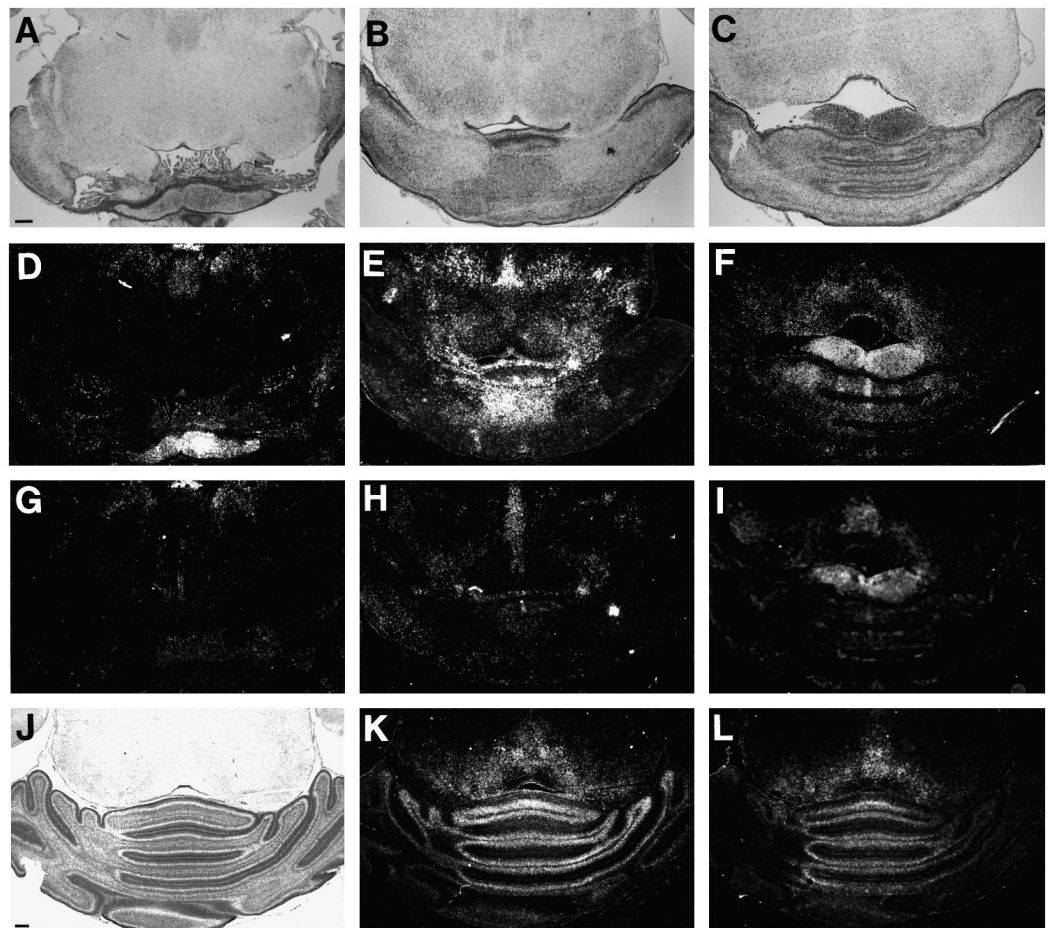


Fig. 5. *Engrailed* expression in the developing postnatal cerebellum. Ventral (A,D,G), medial (B,E,H) and dorsal (C,F,I) bright-field (A-C) and dark-field (D-I) micrographs of horizontal sections through P0 cerebella probed with *En-2*-specific (D-F) and *En-1*-specific (G-I) probes. *En-2* expression was limited to the developing vermis and bands of higher expression were only faintly detected in dorsal sections (F). *En-1* expression could not be detected. (J,K) Bright- and dark-field micrographs of a medial horizontal section of a P5 cerebellum probed with *En-2*, showing that *En-2* expression was still not detected in the most lateral hemispheres at this stage. (L) An adjacent section probed with *En-1*, indicates that *En-1* expression was also centrally restricted. Anterior is to the top. Scale bars, 100 μ m.

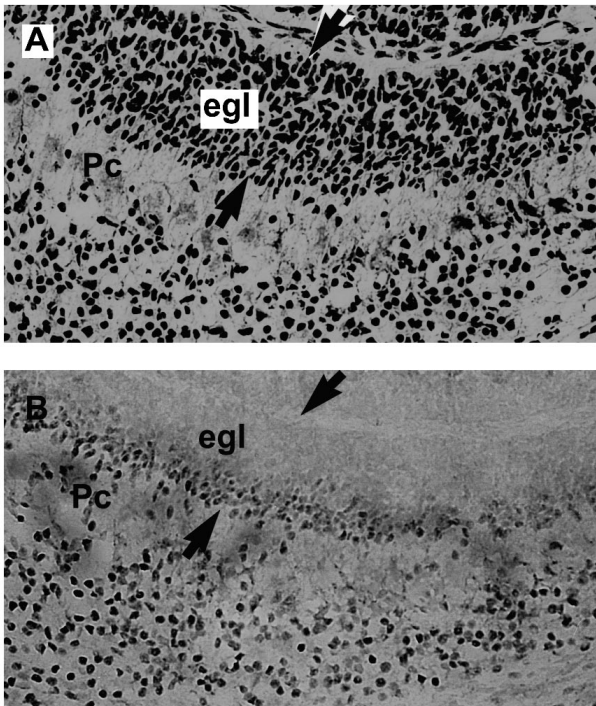


Fig. 6. *En-2* is expressed in the inner migrating cells of the EGL, not the outer proliferating cells. (A) A cresyl violet section through a wild-type P5 cerebellum to demonstrate the cell types of the cerebellum and (B) a similar section stained with the α -*En-2*hb-1 antiserum showing granule cell labelling in the inner layer of the EGL and granule cells migrating to form the internal granule layer. No expression is seen in Purkinje cells. egl, external granule layer; Pc, Purkinje cells.

showed spatial patterning at P2. No medial to lateral differences in expression were evident for any gene in horizontal sections except *En-1* and *En-2* (Fig. 5 and data not shown).

At P2, *Wnt-7B* expression was higher in the anterior of the cerebellum, with a boundary of expression within lobule VI (Fig. 8A). Expression was also observed in lobule X. At both P5 and P8, there appeared to be a down-regulation of *Wnt-7B* expression and the spatial restriction was no longer distinct (Fig. 8C,D). At P2, *Gli* also showed spatial differences in expression, with stronger expression in the anterior of the cerebellum (Fig. 8E). This spatial restriction was diminished by P5 and by P8 transcripts were found across the entire cerebellum (Fig. 8H). Examples of non-patterned gene expression were *Gli-2* and *Gli-3*, which were not detected at P2, but showed strong expression throughout the cerebellum at P5 and P8, in the EGL and an inner cell layer (Fig. 8L,P).

Patterns of gene expression are altered in the *En-2* mutant cerebellum during postnatal development

All genes that were expressed in the wild-type cerebellum at P2 and P5, were also expressed in the *En-2* mutant cerebella at the same stages. Non-patterned postnatal gene expression remained unpatterned in the *En-2* mutant. Further, RNase protection assays to detect *Pax-3* and *Pax-6* message in wild-type, heterozygous or homozygous mutant cerebella at P5 showed no evidence of changes in the steady-state levels of transcripts of these two non-patterned genes (data not shown) in the mutant.

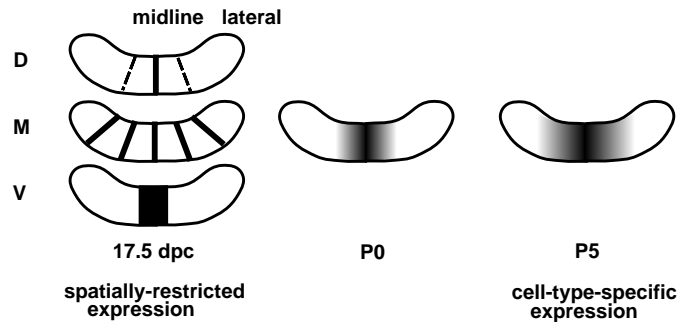


Fig. 7. A summary of wild-type *En-2* expression during cerebellar development. Schematic horizontal sections through cerebella at 17.5 dpc, P0 and P5 are illustrated, with *En-2* expression indicated as shaded regions. D, dorsal; M, medial; V, ventral.

At P2, the *En-2* probe detected expression in the mutant similar to the wild-type pattern, but with the anterior boundary of expression located more posteriorly than in the wild-type. A decrease in lobule X expression was also observed (Fig. 8J). At both P2 and P5, *En-1* expression was not altered from the wild-type expression in the anterior lobules and similar to *En-2* expression, *En-1* lobule X expression was also decreased (Fig. 8N). In the *En-2* mutant cerebellum, although *Wnt-7B* was still expressed at a higher level in the anterior of the cerebellum, the anterior/posterior boundary of expression was shifted to a more posterior lobule (Fig. 8B). No major change was seen for *Gli* expression in the mutants (Fig. 8F). As in wild-type animals, no spatial differences in expression were observed by P8 in the *En-2* mutants.

DISCUSSION

Based on our expression study and the established role of the *Drosophila* segmentation genes in patterning fly embryogenesis, it seems likely that vertebrate homologues of *Drosophila* segmentation genes are responsible for regulating both medial/lateral and anterior/posterior positional information in the developing cerebellum. At least one member of the *En*, *Wnt*, *Pax*, *Gli* and *Dvl* gene families is expressed in the developing mouse cerebellum and furthermore, one member of all but the *Dvl* gene family shows dynamic and spatially restricted gene expression. During late embryogenesis and early postnatal development, both dorsal-ventral as well as medial-lateral spatial differences were observed in expression patterns. Strikingly, at 17.5 dpc, positive and negative regions of *En-2* expression divided the cerebellum into eleven sagittal domains, with one midline band flanked by 5 bilaterally symmetrical sagittal bands (Fig. 3). *En-1* was expressed in three of the *En-2* bands, whereas *Wnt-7B* and *Pax-2* were expressed in the complementary, *En-2*-negative bands. Postnatally, although spatially restricted gene expression was observed, the patterns of gene expression were very different. Along the anterior/posterior axis of the vermis at P2, *Wnt-7B*, *Gli*, *En-2* and *En-1* expression divided the cerebellum into anterior/posterior domains. Each of these genes had higher levels of expression in the anterior of the cerebellum with borders of expression within lobules V and VI (Fig. 8). In the medial/lateral axis, *En-2* and *En-1* were only detected in the vermis, with no hemisphere expression (Fig. 4). By P8, when

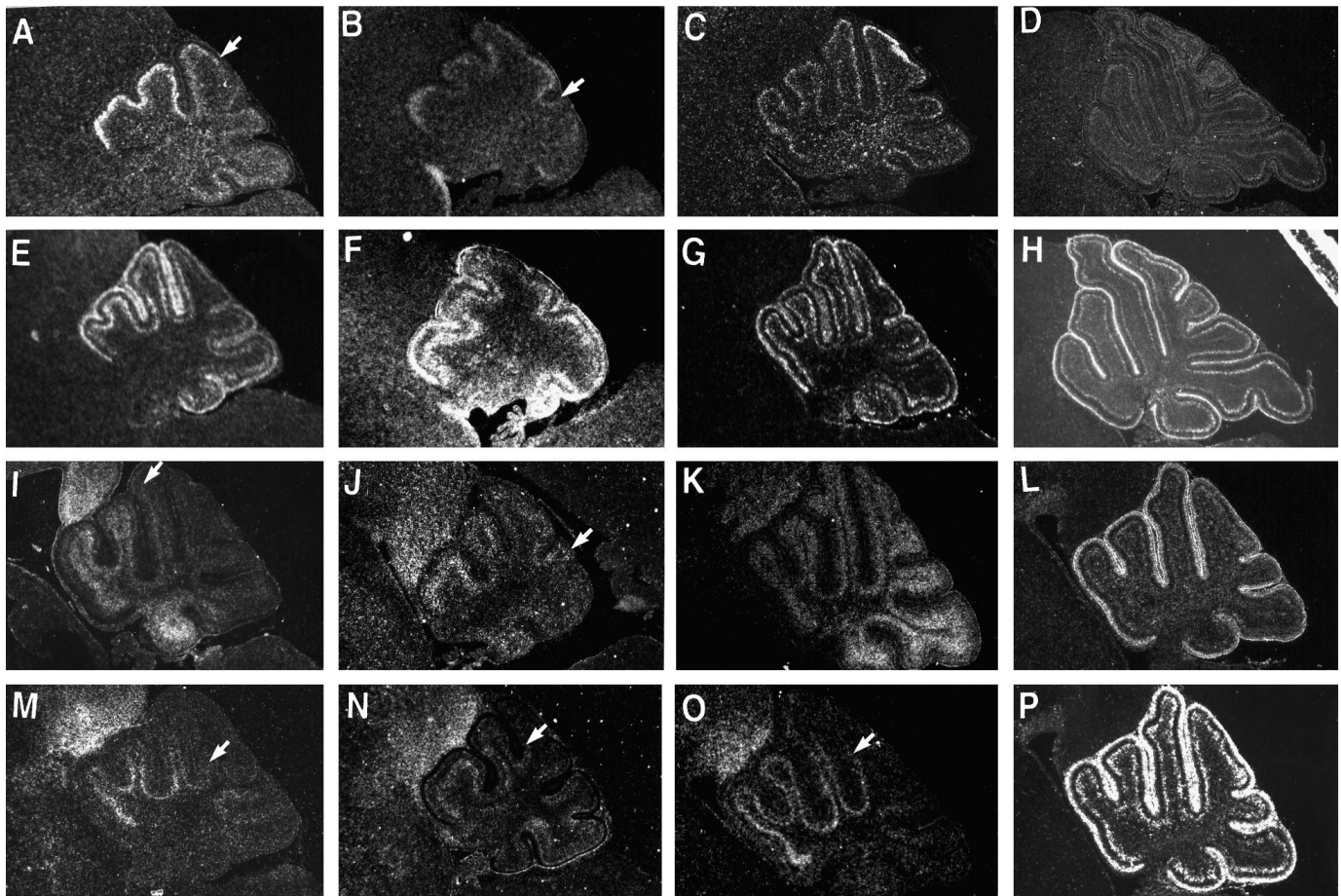


Fig. 8. Gene expression in the postnatal cerebellum is dynamic. Mid-sagittal sections of wild-type P2 (A,E,I,M); *En-2^{hd}* homozygous mutant P2 (B,F,J,N); wild-type P5 (C,G,K,L,O,P) and wild-type P8 (D,H) cerebella. Anterior is to the left, dorsal is to the top. (A-D) *Wnt-7B* expression at P2 had a distinct boundary of expression at P2 in the wild-type cerebellum (arrow in A) in lobule VI which was shifted to a more posterior position in the P2 mutant cerebellum (arrow in B). By P5, there was no strong spatial restriction of expression (C) and by P8, *Wnt-7B* was barely detectable (D). (E-H) *Gli* was expressed more strongly in the anterior lobules at P2 in the wild-type (E). No major change in expression pattern was seen in P2 *En-2^{hd}* homozygous mutant cerebella (F). By P8, no spatial differences in wild-type expression were observed (H). (I-K) In the wild-type, *En-2* was expressed in both anterior and posterior lobules at P2 (I) and at P5 (K) there was a clear gradient of *En-2* expression, with higher levels found posteriorly. This pattern was changed in the mutant at P2 (J). (M-O) *En-1* had anteriorly-restricted expression at both P2 (M) and P5 (O), which was not altered in the *En-2* mutant (N). *Gli-2* (L) and *Gli-3* (P) are examples of genes that show no spatially restricted expression at P5. Arrows in I, J, M-O indicate boundaries of gene expression. Scale bars, 100 μ m.

the basic adult pattern of foliation and afferent input is established, no spatial differences were seen in expression of any gene examined. Consistent with these genes regulating establishment of the spatial cues required for foliation patterning, patterned gene expression was altered in *En-2^{hd}* mutants at both 17.5 dpc and P2 (Figs 2, 8).

***En-2* patterned expression and the *En-2* mutant foliation defect**

The expression patterns of both *En-1* and *En-2* during late embryonic and early postnatal cerebellar development provide a possible interpretation of the basis of the patterning defect observed in the *En-2* mutant cerebellum. The *En-2* cerebellar phenotype is characterized by a general developmental delay and size reduction that is evident as early as 15.5 dpc. There are also distinct foliation patterning defects in both the vermis and the hemispheres that arise from abnormal formation of fissures during early postnatal development. A time-course of *En-2* expression is outlined in Fig. 7. At both 15.5 and 17.5

dpc, *En-2* was expressed across the cerebellum with higher levels of patterned expression most obvious in the medial horizontal plane. At birth, *En-2* was no longer expressed in the hemispheres of the cerebellum and hemisphere expression was not detected again until P8, after abnormal fissure formation occurs in the mutant hemispheres (summarized in Fig. 7). This suggests that the last possible time that *En-2* could exert any direct influence on hemisphere foliation is at 17.5 dpc. Furthermore, postnatal *En-2* expression was not found in Purkinje cells but was limited to granule cells and cells of the molecular layer. In addition, high levels of En protein were not found in the outer proliferating EGL cells, but in the inner migrating layer of the EGL. Since foliation is thought to arise from differential EGL proliferation (Mares et al., 1970; Mares and Lodin, 1970) and not EGL cell migration, our expression studies indicate that *En-2* is not likely to be directly involved in the postnatal process of foliation. Rather, *En-2* likely plays an earlier role in setting up the folial pattern during late embryogenesis, around 17.5 dpc.

Our analysis of *En-1/En-2* compound homozygous mutants and animals in which *En-1* is replaced with *En-2*, demonstrate that *En-1* and *En-2* can compensate for each other's function in cells where both genes are expressed (Hanks et al., 1995; Wurst et al., unpublished data). It was therefore important to examine the expression of *En-1* to determine in which cells *En-1* could be compensating for the lack of *En-2* function. During cerebellar development, *En-1* was only detected in a single sagittal band spanning the mid-line at 15.5 and 17.5 dpc and was not detected postnatally until P2 at the midline. Thus, *En-1* could only contribute to cerebellar patterning at the cerebellar mid-line.

Spatially restricted gene expression patterns and cerebellar foliation

The spatially restricted sagittal bands of expression observed at 17.5 dpc for *En-2* and the other genes implicated in folial patterning are particularly intriguing in light of evidence that there is intrinsic medial/lateral and anterior/posterior patterning information in the embryonic cerebellar anlage at this stage. Genes such as the Purkinje cell-specific proteins calbindin and *L7/pcp-2* are induced in parasagittal bands of Purkinje cells in a temporally and spatially reproducible manner, during late embryogenesis and early postnatal development (Oberdick et al., 1990; Vandaele et al., 1991 and Wassef et al. 1992a). Although expression of such genes are thought to reflect an intrinsic topographic map of the cerebellum, until now, no candidate molecules have been described that could establish this positional information in the developing cerebellum. Our studies provide the first evidence of likely candidate genes regulating spatial cues in the developing cerebellum.

Strikingly, the expression patterns of *En-2*, *Wnt-7B* and *Pax-2* at 17.5 dpc apparently divide the cerebellum into a set of eleven similar sagittal domains, suggesting that these eleven domains represent fundamental patterning elements of the embryonic cerebellum. We have defined these domains as a midline domain A and 5 bilaterally symmetrical domains B-F. Using this nomenclature, the *En-2*-expressing domains are A, C and E, while both *Wnt-7B* and *Pax-2* are expressed in the complementary domains B, D and F (Fig. 3). Whether other regulatory genes will mark domains that are out of register with these eleven domains remains to be determined. From our *in situ* data it is not possible to determine if any of the boundaries of gene expression exactly coincide. A likely 'fuzziness' of domain boundaries could add to the level of complexity of the positional information encoded within the developing cerebellum. There is, however, evidence that there is a complexity of positional information encoded within each sagittal domain. For example, *En-1* expression is narrower than *En-2* expression in domains A and C. As well, the *Wnt-7B* expression in band B is restricted to the posterior half of the band, unlike *Pax-2* which is expressed in both the anterior and posterior regions of this band.

Alternating on/off gene expression as a genetic mode of establishing domains is reminiscent of the stripes of expression of the pair-rule set of segmentation genes across the epidermis in the early *Drosophila* embryo. Segment polarity genes such as *en*, *wnt* and *ci* are activated later in subsets of cells in each segment, defining the anterior or posterior compartments. At 17.5 dpc, the expression of *En-2*, *Wnt-7B* and *Pax-2* seem to

be analogous to the early pair-rule genes, defining eleven domains and looking very much like an early *Drosophila* embryo. In contrast, in the earlier neural tube, *En*, *Wnt* and *Pax* genes are co-expressed in one mid/hindbrain region. One difference between these two stages of vertebrate CNS development is that the early mid/hindbrain region of the neural tube undergoes extensive expansion through proliferation whereas the embryonic cerebellum is primarily composed of postmitotic neuronal cells. This latter situation is more akin to the *Drosophila* embryo during segmentation, where cell signalling is occurring between cells which have already undergone a phase of proliferation.

Postnatally, we observed anterior/posterior differences in the wild-type expression patterns of *En-1*, *En-2*, *Wnt-7B* and *Gli*, as well as medial/lateral differences in *En* postnatal expression. Transgene expression, fate mapping and phenotypic analysis of the *meandertail* cerebellar mutant have provided evidence that the developing cerebellum can be divided into anterior and posterior developmental domains, with the compartment boundary in the VIth lobule (Logan et al., 1992; Oberdick et al., 1993; Hallonet et al., 1990; Ross et al., 1990). This is the same lobule where we observed a restriction of expression of *Wnt-7B* and *Gli* at P2. *En-1*, *En-2* and *Wnt-7B* also marked other borders. In particular, *Wnt-7B* and *En-2* mark a similar border in lobule IX which may correspond with a later zebrin boundary (Tano et al., 1992). Interestingly, anterior/posterior boundaries of *Otx-1* and *Otx-2* expression also have been identified within the postnatal cerebellum, possibly marking major functional boundaries of the cerebellum (Frantz et al., 1994). Our results thus provide further support to the hypothesis of anterior/posterior compartmentalization of the developing cerebellum and provide candidate genes for maintaining such boundaries. It is interesting to note that the expression defining anterior/posterior regions in the postnatal cerebellum is reminiscent of expression of the *Drosophila* homologues of these genes in the fly imaginal disk development. At this later stage of *Drosophila* development, unlike the reiterated bands of gene expression seen during the earlier segmentation of the fly epidermis, large domains of gene expression define two compartments.

Genetic interactions during cerebellar patterning

Expression of the patterned genes was examined in *En-2* mutant cerebella to determine whether their expression was altered in a folial patterning mutant and to uncover possible genetic interactions. A schematic representation of this data is included as Fig. 3. Transcripts from the *En-2* locus demonstrated that *En-2*-expressing cells were not deleted in the *En-2* mutant, but instead the pattern of *En-2* expression was altered. At 17.5 dpc, instead of the wild-type five sagittal bands of expression, there was one broadened midline band with two partial lateral bands that did not appear to correspond in position with either the C or E bands. The *En-1* bands of expression seemed to be lost or became more diffuse. In contrast, the basic pattern of *Pax-2* expression remained in *En-2* mutants, although the size of the expressing bands B and D were reduced and F expanded. *Wnt-7B* had the most striking alteration in expression pattern, with the pairs of expressing bands B and D being lost. The nearly normal *Pax-2* expression indicates that band D cells are present in the mutants but fail to express *Wnt-7B*. This suggests that *Pax-2* expression is inde-

pendent of *En-2* function, whereas *En-1* requires *En-2* function. Expression of *Wnt-7B* in bands B and D is dependent on *En-2* function in bands A, C and D. Based on the positions of cells which express the above genes, *En-2* may directly regulate expression of *En-1* whereas the influence of *En-2* on *Wnt-7B* expression must be indirect. Careful examination of the patterns of *En-2* and *Wnt-7B* indicate that the wild-type patterns are complementary in fine detail. The possibility that *En-2* is required to maintain *Wnt-7B* expression through cell-cell interactions is particularly interesting since this is the case in the fly epidermis for *en* and *wg* (Hooper and Scott, 1992). Our data also allow us to conclude that although *En-2* function may be required for the correct size and spacing of cerebellar domains A-F, it is not essential for formation of the domains, since in *En-2* mutants *Pax-2* expression still delineates the eleven sagittal bands.

In the *En-2* mutant cerebellum at P2, the anterior/posterior boundary of expression of *Wnt-7B* expression was shifted to more posterior positions, suggesting a posterior to anterior change in positional identity. This is precisely what was observed with two transgenes showing boundaries of expression in this region of the adult cerebellum (Millen et al., 1994).

Downstream genes

Genes encoding calbindin and *L7/pcp-2* and other genes transiently expressed in sagittal domains in the late embryonic and early postnatal cerebellum seem to be excellent candidates for genes downstream of *En-2* and/or the other patterning genes identified in this study. Our preliminary analysis of calbindin expression relative to *En-2* at 17.5 dpc appears to indicate that both genes are expressed in overlapping, but not-identical domains (data not shown). Comparing our data with that of Oberdick et al. (1993), *pcp-2/L7* appears to be expressed in the *En-2*-negative bands B, D and F at 17.5 dpc. Transgenic studies of upstream regions of the *L7* gene have revealed regulatory elements that are sensitive to the position of Purkinje cells and which direct expression that respects medial-lateral and anterior-posterior boundaries within the developing cerebellum in a grid-like manner (Oberdick et al., 1993). It is of interest to note that homeobox-binding sites have been identified upstream of the *L7/pcp-2* gene and that these sites are thought to be important in regulating the segment-like patterns of *L7/pcp-2* gene expression in the developing cerebellum (Oberdick et al., 1993). It will be interesting to determine whether *L7/pcp-2* gene expression is altered in the *En-2* mutant.

Implications for mechanisms of cerebellar patterning

The genes described in this study meet the criteria of being expressed at the right time and in the right place to be regulating fundamental spatial cues for cerebellar patterning. Postnatal gene expression was seen to divide the cerebellum into anterior and posterior compartments, similar to those previously documented in mutants. At 17.5 dpc, a complex network of positional information encoded by spatially restricted gene expression of vertebrate homologues of *Drosophila* segment polarity genes define eleven sagittal domains across the cerebellum. Interestingly, the expression is across all cell types. These domains of regulatory gene expression are similar to

domains of gene expression of Purkinje cell markers such as calbindin and *L7/pcp-2*. Our analysis indicates that at least *En-2* and *En-1* mark spatially restricted regions of the cerebellum from at least 15.5 dpc. Earlier studies have shown that Purkinje cells have intrinsic positional information by 14 dpc (Oberdick et al., 1993). Until this study, transient, spatially restricted gene expression in Purkinje cells of the late embryonic and early postnatal developing cerebellum was suggested to be important for afferent patterning (Wassef et al., 1992a, b). Consistent with this, we have evidence that in addition to foliation, mossy-fiber patterning is altered in *En-2* mutants (Vogel et al., unpublished). Taken together, the various expression studies and mutant analysis indicates that this group of homologues of segmentation genes could play key roles in regulating afferent patterning in the cerebellum.

The foliation defect in *En-2* mutants and our present results also implicates spatially restricted gene expression in patterning of cerebellar folia. Based on the expression patterns, much of this must be set up in the late embryonic cerebellar anlage. These genes may regulate postnatal foliation through the control of regional differentiation of Purkinje cells and other postmitotic neurons that, among other things, determines the later expression of sets of cell surface molecules involved in cell migration. They may also have long term effects on postnatal proliferation of EGL cells. Alternatively, since there is extensive evidence for Purkinje cell-granule cell interactions during cerebellar development, these genes may serve to set up position-dependent signals in Purkinje cells that postnatally influence proliferation and/or migration of EGL cells. One final alternative is that the embryonic spatial cues only directly influence afferent patterning and that it is the position of the afferents that direct the pattern of foliation postnatally.

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