Abnormal embryonic cerebellar development and patterning of postnatal foliation in two mouse Engrailed-2 mutants

Kathleen J. Millen², Wolfgang Wurst², Karl Herrup³ and Alexandra L. Joyner¹,²,*

¹Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada
²Department of Molecular and Medical Genetics, University of Toronto, Canada
³Alzheimer Research Laboratory, Case Western Reserve, Cleveland, Ohio, USA

*Author for correspondence at address 1

SUMMARY

The cerebellum is an ideal system to study pattern formation in the central nervous system because of its simple cytoarchitecture and regular organization of folds and neural circuitry. Engrailed-2 (En-2) is expressed in a spatially restricted broad band around the mesencephalic-metencephalic junction, a region from which the cerebellum is derived. Mice homozygous for a targeted deletion of the En-2 homeobox, En-2ₜₙ, previously have been shown to have an altered adult cerebellar foliation pattern. To address whether the En-2ₜₙ allele was hypomorphic, we generated a putative null mutation that makes an N-terminal deletion (ntd). Mice homozygous for this new mutation, En-2ₜₙ, display an identical cerebellar patterning defect, suggesting that both alleles represent null alleles. We also examined the developmental profile of En-2 homozygous mutant cerebellar foliation. This revealed a complex phenotype of general developmental delay and abnormal formation of specific fissures with the most severe morphological disruptions being limited to the posterior region of the cerebellum. The expression of two transgenes, which express lacZ in lobe-specific patterns in the cerebellum, also was found to be altered in En-2 homozygotes, suggesting possible lobe transformations. Finally, during embryogenesis there was a clear delay in fusion of the cerebellar rudiments at the midline by 15.5 d.p.c. This and the expression pattern of En-2 suggests that although cerebellar foliation is largely a postnatal process, the patterning of the cerebellum may begin during embryogenesis and that En-2 plays a critical role in this early process.

Key words: cerebellum, development, foliation, patterning, homeobox

INTRODUCTION

The cerebellum is particularly amenable to the analysis of pattern formation because of its regular organization. Structurally, the cerebellum is one of the simplest structures of the CNS. It is composed of only a few cell types, all organized in a precise fashion in distinct morphological layers (reviewed by Llinas, 1975). Additionally, the cerebellum has a characteristic set of folds separated by fissures that run perpendicular to the anterior-posterior axis. These folia are stereotypical in structure between animals of the same species and are highly conserved across evolution. In mammals, there is a central set of 8 folds that make up the vermis, two sets of 4 folds that make up the lateral hemispheres and two sets of folds making up the paraflocculus, which is situated most laterally (see Fig. 3A,D). Based on the conservation of these folia and their structure across evolution, Welker (1990) has proposed that each folium acts as an independent processing module carrying out integrative operations on a patterned assortment of afferent inputs that are unique for each folium. Thus, each folium could represent a unique functional domain. There are indications from lineage analysis experiments in the chick (Martinez and Alvarado-Mallart, 1989), mutant phenotypes in the mouse (Ross et al., 1990) and transgene expression in the mouse (Logan et al., 1993) that the cerebellum may also be subdivided into anterior and posterior compartments that cross the vermis and hemispheres.

The process of cerebellar foliation is primarily a postnatal process, although the cerebellar anlage arises during embryogenesis. The first morphological indications of the developing cerebellum are two bilateral thickenings of the roof of the anterior metencephalon that arise between 11 and 12 days post coitum (d.p.c.) (Fig. 1A) (Miale and Sidman, 1961). As development proceeds, these bilateral thickenings expand and grow together, fusing on the midline by 15.5 d.p.c. (Fig. 1B). Granular neuron precursors are derived from a distinct germinal zone in the cerebellar anlage.
(Miale and Sidman, 1961) between 13 and 15 d.p.c. and migrate anteriorly and mediially covering the entire developing cerebellum to form the external granular layer (EGL). These cells remain mitotically active and undergo massive proliferation postnatally. Cerebellar foliation is thought to arise from the differential proliferation of the EGL (Mares et al., 1970; Mares and Lodin, 1970), however, the cellular and genetic regulatory mechanisms responsible for this are unknown.

The existence of several mouse mutations that disrupt the regional organization and foliation of the cerebellum provides one approach to address the genetic basis of cerebellar development. In terms of pattern formation, meandertail and leaner, for example, have a primary effect on specific cell types in the anterior portion of the developing cerebellum and secondarily influence the pattern of cerebellar foliation (Ross et al., 1990; Herrup and Wilczynski, 1982). Only three loci have been identified that specifically affect cerebellar foliation; Cerebellar folial pattern-1 (Cfp-1) (Neumann et al., 1990; Cooper et al., 1991) and decidual sulcus of cerebellum (dsc) (Wahlsten and Andison, 1991), each appear to control the formation of specific sulci in the cerebellum. Neither of these genes has been characterized at the molecular level. The third gene, Engrailed-2 (En-2), has multiple effects on the patterning of cerebellar folia (Joyner et al., 1991).

Members of the engrafted gene family encode highly conserved transcription factors that contain a homeodomain (reviewed by Joyner and Hanks, 1991). During early development, the murine Engrailed genes, En-1 and En-2, are expressed in similar spatially restricted domains across the presumptive mid-hindbrain junction, the region from which the entire cerebellum is derived (Davis and Joyner, 1988; Davis et al., 1988; Davis et al., 1991; Davidson et al., 1988). Later the expression becomes restricted to groups of cells in the colliculi, substantia nigra and pons (Davis and Joyner, 1988). Additionally, En-2 is broadly expressed in the embryonic cerebellum and postnatally becomes restricted to granular cells and cells in the molecular layer (Davis and Joyner, 1988; Davis et al., 1988). To examine En-2 gene function, a mutation, En-2\textsuperscript{hd}, was made that deletes the coding region of the homeobox-containing exon 2 (Joyner et al., 1989; Joyner et al., 1991). Mice homozygous for this mutation are viable and show no gross problems in motor control. However, the animals do exhibit a reduced cerebellar size and a distinct patterning defect of the cerebellar folia. In En-2\textsuperscript{hd} homozygous animals, the folding pattern is relatively normal in the anterior region of the cerebellum whereas it is disrupted in the posterior region. Most notably, in the posterior vermis, there is an apparent realignment of the VIII\textsuperscript{th} fold with the IX\textsuperscript{th} instead of with the VI\textsuperscript{th} and VIP\textsuperscript{th}.

In the hemispheres, only 3 and not the normal 4 folds are present and there is abnormal folding and a dramatic reduction in the size of the paraflocculus (Joyner et al., 1991).

Considering the early spatially restricted broad band of En-2 expression in the neural tube, En-2 mutations might be expected to disrupt more than cerebellar patterning. One possible explanation for the relatively mild En-2\textsuperscript{hd} phenotype is that it represents a hypomorphic allele. Other studies have shown that both ftz and Hoxd-8 have biological activity in the absence of a homeodomain (Fitzpatrick et al., 1992; Ananthon et al., 1993 and Zappavigna et al., 1991). We therefore made a second, predicted null mutation that deleted the first exon. Animals homozygous for this mutation were found to display the same cerebellar phenotype as En-2\textsuperscript{hd} mutants, arguing that the En-2\textsuperscript{hd} allele is non-functional. In order to gain insight into the cellular basis of the patterning defect, we have examined cerebellar development in mice homozygous for the En-2\textsuperscript{hd} mutation during embryogenesis and postnatally. This analysis revealed a complex phenotype of general developmental delay, abnormal fissure formation and possible anterior/posterior transformations of lobe identities. A defect in cerebellar development could be seen clearly as early as 15.5 d.p.c., suggesting that En-2 is required for the generation or specification of cerebellar precursors that are essential for correctly patterning postnatal foliation.

Fig. 1. A schematic diagram of embryonic (A) and postnatal (B) cerebellar development. (A) Parasagittal and transverse views through the mesencephalic-metencephalic junction of the CNS during embryonic development. The cerebellar anlage is indicated in black and the EGL by the stippled line. D, dorsal; V, ventral; A, anterior; P, posterior; EGL, external granular layer. (B) Mid-sagittal views through the developing postnatal cerebellum, showing the progression of foliation and the formation of the internal granular layer (IGL). Anterior is to the left, dorsal is top.
box. Probes used for Southern analysis are as marked. Bg, BgIII; P, PstI; H, HindIII; Xh, XhoI; C, ClaI; S, SstI; E, EcoRI. (B) Southern Blot autoradiograms of PstI digested genomic DNA from offspring of an En-2<sup>ntd</sup> heterozygote cross, probed with the 3<sup>′</sup> probe. Arrows indicate wild-type (wt) and mutant (m) bands. Lanes 1 and 4 show the heterozygous mutant pattern, lanes 2 and 5 show wild-type patterns and lane 3 shows the homozygous mutant pattern. On the left, the migration positions of HindIII-digested λ DNA markers are indicated.

**MATERIALS AND METHODS**

**Gene targeting**

An exon 1 En-2 targeting vector (Fig. 2A) of the positive/negative type (Mansour et al., 1988) was made from genomic DNA from C57 strain mice. The vector contained a neo cassette (Joyner et al., 1989) surrounded by a 0.63 kb HindIII/XhoI genomic fragment containing part of the En-2 promoter and 5′ untranslated sequences (Logan et al., 1992) and a 3.4 kb SstI/HindIII fragment containing a 324 bp of the 3′ end of exon 1 and nearly the entire intron. The MCI tk cassette (Mansour et al., 1988) was inserted 3′ to the long arm.

The SstI linearized targeting vector was electroporated into D3 (Doetschman et al., 1985) embryonic stem (ES) cells, as described by Wurst and Joyner (1993). A total of 5x10<sup>7</sup> ES cells were electroporated with 400 µg of vector DNA and approximately 250 G418<sup>®</sup>/GANC<sup>®</sup> colonies (representing a 10-fold enrichment) were examined by Southern blot analysis for targeted colonies. ES cell DNA was digested with PstI and probed with either a 0.35 kb BglII/HindIII 5′ flanking probe (Fig. 2A) or with a 2.3 kb SstI 3′ flanking probe (Joyner et al., 1991) (Fig. 2A). PstI digestion produced a 7.6 or 8.5 kb wild-type and 5.7 or 1.0 kb mutant band, when probed with the 3′ or 5′ probes, respectively. One colony was identified that had undergone the predicted homologous recombination replacement event and another that had undergone an abnormal insertion event and had undergone an abnormal insertion event (data not shown). ES cells containing the replacement event were injected into C57BL/6J blastocysts (Papaioannou and Johnson, 1993). Three chimeras were found to produce ES cell-derived agouti mice when bred with C57BL/6J females. Approximately fifty percent (19/34) of the agouti offspring were found to be heterozygous for the En-2 exon 1 predicted null allele, En-2<sup>ntd</sup> (N-terminal deletion). All genotyping of animals was done by Southern blot analysis of tail DNA digested with PstI and probed with the 3′ probe (Fig. 2B).

**Mouse strains**

Mice carrying the En-2<sup>hd</sup> allele (Joyner et al., 1991) were maintained on either a CD1 outbred background or a 129Sv inbred background by breeding homozygotes to either heterozygous or homozygous animals. Offspring were genotyped as described by Joyner et al. (1991). For analysis of cerebellar foliation, littermate 129Sv control animals (En-2<sup>2hd/+</sup>) were compared with En-2<sup>2hd/-</sup> homozygous mutant animals at embryonic stages and postnatal day 0 (P0). For later stages, age-matched wild-type control 129Sv animals and En-2<sup>2hd/En-2</sup> animals on the 129Sv background were analyzed.

Two transgenes showing specific cerebellar lacZ staining patterns were transmitted into the En-2<sup>2hd</sup> mutant stock on the outbred CD1 background; β2γ is a transgenic line carrying a pcp-2 promoter/lacZ reporter construct (Vandaele et al., 1991) and Tg5 is a transgenic line carrying an En-2 promoter/lacZ reporter construct (Logan et al., 1993). A lacZ probe was used for Southern blot analysis to identify animals carrying either transgene. Brains of 6-week old littermates from the following cross were examined for lacZ activity either in whole mount or on sections: En-2<sup>2hd/+</sup>; transgene/transgene with En-2<sup>2hd/+</sup>; +/-/+. Eight heterozygous and eight homozygous En-2<sup>2hd</sup> mutants carrying the β2γ transgenes were analyzed. Four heterozygous and four homozygous En-2<sup>2hd</sup> mutants carrying the Tg5 transgene were analyzed.

**Histology**

For histological analysis of brain tissue at embryonic stages 13.5-17.5 d.p.c., intact heads of the embryos were fixed in 4% paraformaldehyde in PBS overnight at room temperature (RT), then transferred to 70% ethanol in saline at RT from several hours to overnight. The brains were then dissected into 70% ethanol. For analysis of newborn to P9 brain tissue, whole brains were dissected directly into Bouin’s fix (BDH), at RT overnight to 2 days, then transferred to 70% ethanol.
For later stages, the brains were bisected sagitally, fixed in 4% paraformaldehyde in PBS for two days at RT and transferred to 70% ethanol. The brains were dehydrated and processed through xylene into wax. Sections were cut at 6-10 µm and counterstained with cresyl violet.

Detection of β-galactosidase activity
Whole brains of animals carrying the β2γ transgene and cryostat sections of cerebella of animals carrying the Tg5 transgene were stained for β-galactosidase activity as described by Gossler and Zachgo (1993).

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 (PBSMT), twice for 5 minutes, followed by a 2 hour RT incubation with a 1:1000 dilution of α-Enhb-1 crude serum (Joyner et al., 1991) in PBSMT. Slides were washed twice for 5 minutes in PBS, 0.3% Triton 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.

RESULTS

En-2 exon 1 deletion mutants have the same cerebellar phenotype as exon 2 homeobox deletion mutants
We used gene targeting in ES cells to generate mice carrying a null mutation of En-2, in which 324 bp of exon 1, including the sequences coding for the 108 N-terminal amino acids, were replaced by a neo cassette. Animals heterozygous for this N-terminal deletion (ntd), En-2<sup>ntd</sup>, on an F1 129/Sv × C57BL/6J background were intercrossed and the expected ratio of wild-type:heterozygous:homozygous animals was obtained (12:31:16) (see Fig. 2B). The homozygous animals were found to be viable, fertile and indistinguishable from their wild-type littermates based on behaviour and overall morphological appearance. To confirm that the En-2<sup>ntd</sup> mutation represented a null allele, immunohistochemistry was performed on cerebellar sections from wild-type and En-2<sup>ntd</sup> homozygous mutant animals, using the αEnhb-1 polyclonal serum, which detects the homeodomains of En-1 and En-2 proteins (Davis et al., 1991). There is no En-1 expression in the adult wild-type cerebellum, while En-2 is highly expressed in the granule cells and cells in the molecular layer. No protein was detected with the antiserum, indicating that no En-2 or ectopically induced En-1 was expressed in the mutant cerebellum (data not shown).

To determine whether the En-2 homozygous animals had a cerebellar defect similar to En-2<sup>hd</sup> mutants, a histological analysis of adult En-2<sup>ntd</sup> homozygous mutant and wild-type littermates was conducted. The En-2 homozygous mutant brains (n=5), as compared to the brains of heterozygous (n=4) or wild-type (n=1) brains had an apparently normal brain except for a reduction in the size of the cerebellum and abnormal patterning of the folds in the cerebellum. The abnormal folding pattern had all the characteristics of the En-2<sup>hd</sup> homozygous mutant cerebellar folding pattern. The branching pattern of the folia of the cerebella of En-2<sup>ntd</sup> homozygous animals in mid-sagittal sections showed the VIII<sup>th</sup> vermal lobe was associated with the IX<sup>th</sup> instead of the VI<sup>th</sup> and VII<sup>th</sup> vermal lobes (Fig. 3D-F). In the hemispheres, only three instead of four major folds were present and the paraflocculus was about half the normal size (Fig. 3A-C). Because En-2<sup>ntd</sup> and En-2<sup>hd</sup> alleles result in the same cerebellar defects and the molecular nature of the En-2<sup>ntd</sup> mutation indicates no residual activity of the protein, both the En-2<sup>ntd</sup> and the En-2<sup>hd</sup> mutations are taken to be null alleles.

A further detailed analysis of the developmental profile of the cerebellar defect was carried out with the En-2<sup>ntd</sup> allele on an inbred 129/Sv background. A histological examination of 17.5 d.p.c., P0 and P7 homozygous mutant cerebella confirmed that the developmental profile was similar for the En-2<sup>ntd</sup> allele.

En-2 is required for normal embryonic cerebellar development
To determine whether the En-2 mutations affected embryonic cerebellar development, we compared the brains of 13.5, 15.5, 16.5 and 17.5 d.p.c. mutant embryos to wild types. In heterozygous crosses of En-2<sup>hd+/+</sup> animals, we previously showed that cerebellum from adult En-2<sup>hd</sup> heterozygotes were indistinguishable from wild-type animals (Joyner et al., 1991). To compare efficiently normal and En-2<sup>hd</sup> homozygous mutant cerebella, heterozygote with homozygote crosses were set up and littermates were examined. To minimize any effect of background genotype on the phenotype, all the analysis was carried out with inbred 129/Sv mice. In En-2<sup>hd</sup> homozygous animals, there was a slight reduction in cerebellar anlage size at 13.5 d.p.c. (data not shown) and a very clear mutant phenotype at 15.5 and 16.5 d.p.c. All En-2<sup>hd</sup> homozygous animals could be distinguished upon dissection at 15.5 d.p.c. and 16.5 d.p.c. by a decrease in the extent of fusion of cerebellar rudiments at the midline and an apparent size reduction of the cerebellum and colliculi (Fig. 4A-D and data not shown).

All animals that displayed an obvious delay in cerebellar rudiment fusion were homozygous for the En-2<sup>hd</sup> mutation (n=9) whereas none of the En-2<sup>hd+/+</sup> littermates (n=10) displayed this phenotype. By 17.5 d.p.c., no difference between the wild-type and mutant cerebella could be distinguished: the cerebella of homozygous mutants were fused at the midline and any size difference was not immediately obvious in whole mount.

Sectioning of the cerebella at all stages revealed that the cellular organization of the existing anlage was not grossly affected in the En-2 mutants with EGL, Purkinje and deep nuclei cells being present in the normal regions (Fig. 4E,F).

The developmental profile of foliation in the hemispheres revealed the absence of the paramedial fissure
Based only on the structure of the adult cerebellar folds, it was not possible to identify which folds remained and which were abnormal in En-2<sup>hd</sup> mutant cerebella. Because no cerebellar lobe-specific genetic markers have been defined that would assist in the definition of the phenotype, we analyzed the developmental profile of fold formation in wild-type and En-2 mutants. A detailed analysis of the development of cerebellar foliation in the rat (Larsell, 1952) was used as a basis of folia and fissure identification during mouse cerebellar foliation.
Fig. 3. Adult En-2 cerebellar phenotype. (A–C) Whole mount (A) wild-type, (B) En-2<sup>hd</sup> homozygous mutant and (C) En-2<sup>ntd</sup> homozygous mutant adult cerebella. Anterior is to the top of each photograph. Vermis lobes are indicated by roman numerals. The V<sup>th</sup> lobe is the only anterior vermis lobe visible. Posterior lobes VI–IX are indicated. The lobes of the hemispheres are indicated on the right-side of each photograph; S, simplex; c, crus I; cII, crus II; PML, paramedial lobe; p, paraflocculus. Fissures of the vermis and hemispheres are indicated on the left of each photograph, with small letters; 1, primary fissure; sp, superior posterior fissure; i, intercrural fissure; py, prepyramidal fissure; 2, secondary fissure. (D–F) Cresyl-violet stained mid-sagittal sections of (D) wild-type, (E) En-2<sup>hd</sup> homozygous mutant and (F) En-2<sup>ntd</sup> homozygous mutant adult cerebella. Anterior is to the left and dorsal is to the top of each photograph. Vermis lobes are indicated by roman numerals, III–X at the crown of each folium. Fissures are indicated by small letters. Abbreviations are as above, with the addition of; ce, precentral fissure and cu, preculminate fissure.
Because of slight strain-to-strain variation in the foliation pattern (Inouye and Oda, 1980), we included an analysis of the 129/Sv inbred strain. Four to five wild-type and En-2<sup>hd</sup> homozygous animals were collected at a standard time for each day of postnatal development from birth to P9 as well as for P14. The brains were sectioned sagitally and photographs of the cerebellar regions were taken at selected intervals from the mid-line out through the hemispheres (approximately 10-20 sections). All data were consistent between animals for each day of development, with very little variation in the progression of foliation between animals of the same genotype and age.

We have previously shown that in the adult En-2<sup>hd</sup> mutant cerebellum, only three lobes are present in the hemispheres, as compared to four in the wild-type cerebellum (Joyner et al., 1991). A developmental profile of hemisphere foliation was carried out to determine the identity of the mutant hemispheric lobes. The adult wild-type cerebellar hemispheres contain four fissures: the superior posterior, intercrural, ansoparame-dial and the parafloccular fissures. These fissures define the hemispheric lobes: simplex, crus I, crus II and paramedial lobe and separate the paramedial lobe from the paraflocculus. In more medial sections the primary fissure is also present (Fig. 5). In the 129/Sv cerebellar hemispheres, the vermal primary and parafloccular fissures were well defined at P0 and the superior posterior and intercrural fissures were distinguishable. These fissures became progressively more defined as development progressed (Fig. 5C,E,G,I). On P6, the ansoparame-dial fissure become visible separating crus II from the paramedial lobe (Fig. 5E). On P9, the anterior lobe, the simplex, was divided by a small sulcus (data not shown).

The initial patterning of foliation in the hemispheres of the En-2<sup>hd</sup> mutant cerebellum did not appear to be severely disrupted, although overall fissure formation was slightly delayed (Fig. 5B,D,E,H,J). The major patterning defect in the mutant cerebellar hemisphere was the failure of the ansoparame-dial fissure to form; this fissure should separate crus II from the paramedial lobe (Fig. 5F,G,J and data not shown). As development progressed beyond P7 in the mutant, crus II remained fused to the paramedial lobe (Fig. 5J). Also, the most anterior sulcus in the simplex did form at its expected time, P9 (data not shown).

The developmental profile of vermis foliation in En-2<sup>hd</sup> mutant cerebella reveals a complex phenotype of general delay and abnormal fissure formation

A summary of the midline vermis sections is presented in Fig. 6. The anterior region of the adult wild-type cerebellum contains three major vermal fissures: the precentral, preculminate and primary fissures, from anterior to posterior, which define three anterior cerebel-lar lobes: III, IV and V. The posterior of the vermis has four defined fissures: the intercrural, prepyramidal, secondary and posterolateral, from anterior to posterior. These define five posterior cerebellar lobes: VI, VII, VIII, IX and X. (Fig. 3A,D,C).

At birth (P0), the majority of the final vermal fissures were already visible in the 129/Sv wild-type cerebellum (Fig. 6A). They included the preculminate and primary fissures in the anterior region and the prepyramidal, secondary and posterolateral fissures in the posterior region. As development proceeded, these fissures became deeper and all the fissures were clearly established at P7 (Fig. 6I). The most anterior fissure, the precentral fissure, initially appeared at P2 (data not shown) and in the posterior, the intercrural, appeared at P7. Little change in the wild-type pattern of folds was observed after P7, although the external granular layer continued to proliferate and became less distinct. By P14, the external granular

![Fig. 4. Embryonic En-2 phenotype. (A,B) Whole-mount 16.5 d.p.c. brains of (A) wild-type and (B) En-2<sup>hd</sup> homozygous mutant littermates. (C-F) Frontal sections through the cerebellum of 15.5 d.p.c. and 17.5 d.p.c. (C,E) wild-type and (D,F) En-2<sup>hd</sup> homozygous littermates, stained with cresyl violet. Scale bars, 1 µm. Arrows indicate the extent of fusion of the cerebellar rudiments at the midline. Abbreviations are as follows: c, colliculi; cb, cerebellum; h, hindbrain; EGL, external granule layer; P, Purkinje cell layer; DN, deep cerebellar nuclei.](https://example.com/fig4)
layer was dispersed, the internal granular layer complete and the Purkinje cell layer rearranged to a monolayer (data not shown).

The vermis foliation profile of the En-2<sup>hd</sup> homozygous cerebellum revealed a complex phenotype of general developmental delay and abnormal fissure formation that was primarily in the posterior region. At birth, two anterior fissures, the preculminate and primary fissures were visible, although they were less pronounced than in wild-type animals (compare Fig. 6A and B). In general, the anterior region of the mutant cerebellum appeared to develop normally postnatally, except the precentral fissure, initially defined at P2 in the wild-type, was not formed until P6 in the mutant cerebellum (Fig. 6H).

In the posterior region of the mutant cerebellum, only the posterolateral fissure was visible at P0 and it was not as pronounced as in the wild-type (Fig. 4A,B). Development of this fissure appeared to proceed normally. In contrast, the remaining 3 posterior fissures of the homozygous En-2<sup>hd</sup> mutants developed abnormally. No fissure was clearly distinguishable in the mutant between the primary and posterolateral fissures until P2 (data not shown). By P2, a fissure was well established and was clearly seen at P3 (Fig. 6D). A second more posterior fold did not form in this region until P4 (data not shown) and was well established by P5 (Fig. 6F). There are two means of identifying the fissures that formed at P2 and P4 in the mutants. If the usual sequence of fissure formation were conserved, the P2 fissure would be the secondary fissure, misplaced to a more anterior position; the usual positions of the secondary and prepyramidal fissures would then be reversed. Alternatively, based on fold position, the fissure formed at P2 may correspond to the prepyramidal fissure, which is normally visible by P0 and the fissure forming at P4 may be the secondary fissure which is normally visible at 17.5 d.p.c. If the latter is the case, then formation of the secondary fissure is dramatically delayed in the mutants, the secondary fissure is unusually shallow and the prepyramidal fissure is unusually deep in the En-2 mutants.

We will refer to the fissure forming at P2 as the prepyramidal/secondary (py/2) fissure and the fissure forming at P4 as the secondary/prepyramidal (2/py) fissure.

As foliation progressed in the En-2<sup>hd</sup> mutant, there was a

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**Fig. 5.** Development of hemisphere foliation. Sagittal sections through the cerebellar hemispheres of 129/Sv wild-type (A,C,E,G and I) and En-2<sup>hd</sup> homozygous mutant (B,D,F,H and J) animals showing the progression of foliation during postnatal days P0-P14. Anterior is to the left, dorsal is top of each photograph. Hemispheric lobes are indicated as follows; S, simplex; cI, crusI; cII, crusII; PML, paramedial lobe. Fissures in the hemispheres are labelled as follows; p, primary; sp, superior posterior; i, intercrural; a, ansoparamedial; pa, parafloccularis. Scale bars, 1 µm.
clear disruption in formation of the 2/py fissure. Although this fissure was well established at P5 of development, it consistently regressed at P6, reappeared at P7 and then remained (Fig. 6 F,H,J). A careful examination of the sections revealed that at P5 (n=3), the fissure extended across the entire vermis and hemispheres. At P6 (n=3), there was no trace of the fissure in either the vermis or hemispheres and at P7, two out of three cerebella showed the fissure extending once again across vermis and hemispheres at a depth equivalent to that seen at P5 (data not shown). In the third animal at P7, the fissure could only be found in the hemispheres and lateral vermis and had not yet extended across the midline (data not shown). Another feature of the vermal phenotype of the En-2<sup>hd</sup> mutants was the precocious appearance of the intercrural fissure at P5 2 days earlier than in the wild-type (Fig. 6F,I).

The major consequence of the abnormal patterning of fissure formation in the En-2 mutant cerebellum in the vermis was the association of the VIII<sup>th</sup> lobe with the IX<sup>th</sup>. Also, the IX<sup>th</sup> lobe was grossly abnormal in shape and the size of the VI<sup>th</sup> and VII<sup>th</sup> lobes was dramatically reduced (Figs 3D-F, 6K,L). The delay of fissure formation in the anterior cerebellum and the precocious formation of the intercrural fissure did not have such dramatic morphological consequences.

Consistent with the developmental delay of foliation in the En-2 mutants, there appeared to be a delay in IGL formation. This was most clearly seen at P7 where the density of the wild-type IGL appeared much greater than that in the mutant. By P14, a difference was not obvious (Figs 5G,H,I,J, 6I,J and data not shown). There was not,

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**Fig. 6. Development of vermis foliation.** Mid-line sagittal cresyl-violet stained sections of 129/Sv wild-type (A,C,E,G,I and K) and En-2<sup>hd</sup> homozygous mutant (B,D,F,H,J and L) cerebella showing the progression of foliation during postnatal days P0-P9. Anterior is to the left, dorsal is to the top of each photograph. Posterior vermis lobes are indicated by roman numerals, VI-X. Fissures are indicated as follows; ce, precentral; cu, preculminate; 1, primary; i, intercrural; py, prepyramidal; 2, secondary; po, posterolateral. Scale bars, 1 μm.
however, a corresponding decrease in the thickness of the EGL in \(En-2\) mutant cerebella between 15 d.p.c. and P9 as compared to wild type (data not shown).

**Lobe-specific \(\text{lacZ}\) expression reveals possible lobe transformations in the \(En-2\) mutant cerebellum**

To aid in the identification of lobes in the \(En-2\) mutants, two transgenic lines expressing \(\text{lacZ}\) in specific patterns in the cerebellum were bred with \(En-2\) mutant animals. Due to the site of transgene integration, each of the transgenic lines fortuitously displays a unique lobe-specific \(\text{lacZ}\) expression pattern in the adult cerebellum. The two different patterns were consistently and specifically altered in the \(En-2\) mutant animals.

\(\beta 2\) is a transgenic line carrying a \(\text{pcp-2}\) promoter fragment expressing \(\text{lacZ}\). The wild-type \(\text{pcp-2}\) gene is normally expressed in all Purkinje cells; however, in the \(\beta 2\) transgenic line, \(\text{lacZ}\) expression is restricted to groups of Purkinje cells within particular lobes (Vandaele et al., 1991). Because \(En-2\) is not expressed in adult Purkinje cells (Davis and Joyner, 1988), any effect on the \(\text{pcp-2}\) promoter driving \(\text{lacZ}\) expression in \(En-2\) adult mice therefore would not be directly due to lack of \(En-2\) protein in Purkinje cells. Furthermore, since the Purkinje cell density is not obviously affected in the \(En-2\) mutant (Joyner et al. 1991), any change in pattern would reflect an alteration in which cells express the gene, not a deletion of these cells.

Four regions of the cerebellum showed altered staining patterns in \(En-2\) mutants (Fig. 7A,B). In the wild-type cerebellar hemispheres, a large patch of \(\text{lacZ}\)-positive cells was consistently seen in crus I. A similar large patch of \(\text{lacZ}\) staining was never observed in the hemispheres of the \(En-2\) homozygotes, indicating that crus I was abnormal in the mutants. Scattered staining was seen in lateral regions of crus I, crus II and the paramedial lobes of \(En-2\) mutant animals; however, here staining did not show a consistent pattern in either wild-type or mutant animals.

The paraflocculus also showed altered staining in the mutant. In wild-type mice, only the most anterior tip showed strong \(\beta\)-galactosidase activity, whereas in the \(En-2\) mutants, strong staining was seen across the entire paraflocculus, suggesting that cells of the posterior paraflocculus may have taken on an anterior identity.
In the vermis of the wild-type cerebellum, the IXth lobe displayed three longitudinal bands of LacZ staining, parallel to the anterior/posterior axis and diffuse staining was seen in the adjacent VIIIth lobe. In the mutant cerebellum, three bands of lacZ-expressing Purkinje cells extended throughout both lobe IX and VIII. This is consistent with the mutant developmental foliation profile of the vermis, which indicated that the VIIIth lobe had become associated with the IXth (Fig. 6K,L) and suggests that the VIIIth lobe may have adopted the more posterior fate of the IXth lobe.

In wild-type animals, the anterior half of the IVth lobe had two longitudinal bands of lacZ expression, whereas the posterior half of lobe VI and the adjacent VIIth folium had a single broad band of lacZ staining. In mutant animals, only the VIIth lobe had a broad band of staining, with the entire VIth lobe showing two parallel stripes of lacZ staining (Fig. 7B). This result suggests that the posterior region of lobe VI has changed its identity to an anterior lobe VI phenotype. This conclusion is also supported by the altered LacZ staining pattern of the Tg5 transgene in En-2<sup>nh2</sup> mutant animals.

Tg5 is a transgenic line carrying an En-2 enhancer and promoter driving the lacZ gene (Logan et al., 1993). In the adult cerebellum, this promoter normally expresses uniformly in the entire granular layer, as well as in cells of the molecular layer. However, due to the site of integration of the construct, this transgenic line expresses lacZ at higher levels in the granular layer of the posterior versus the anterior vermis. On the midline, there is a sharp border between strong and weak lacZ staining within the granular layer and this coincides with the anterior/posterior boundary of the cerebellum defined by the meandertail mutant (Logan et al., 1993). This boundary lies in the middle of the posterior half of lobe VI. In Tg5 transgenic animals heterozygous for En-2<sup>nh2</sup>, as expected, the boundary between strong and weak lacZ expression was within the posterior half of the VIth lobe, anterior to the intercrural fissure (Fig. 7C). In contrast, the boundary in the En-2<sup>nh2</sup> homozygous mutants was between the VIth and VIIth lobes, at the base of the intercrural fissure (Fig. 7D). This result provides further evidence that the posterior region of lobe VI has properties of the anterior lobe VI in the En-2<sup>nh2</sup> homozygous mutant animals.

**DISCUSSION**

We have made two En-2 targeted mutant alleles and found that both display the same set of defects in cerebellar foliation. Analysis of the development of cerebellar foliation in mutant and wild-type animals revealed the morphological basis responsible for the adult En-2 mutant phenotype. In the En-2 mutants during embryogenesis, a delay in cerebellar fusion was evident by 15.5 d.p.c. as well as an apparent reduction in the size of the cerebellum and colliculi. Postnatally, the patterning of cerebellar foliation showed complex alterations in the En-2 mutant cerebellum, in addition to a general developmental delay. The patterning defects included a lack of formation of one fissure and grossly abnormal formation of two other fissures in the posterior region of the cerebellum. Further changes in regional cell identities in the posterior region, that may reflect lobe transformations, were revealed by analysing posterior lobe-specific patterns of lacZ transgene expression in En-2<sup>nh2</sup> mutants. It is intriguing to note that all the major morphological defects and possibly the transformations associated with the En-2 phenotype were limited to the posterior region of the cerebellum, similar to engrafted mutations in the fly where defects are limited to posterior compartments (Lawrence and Morata, 1976; Kornberg, 1981).

The morphological and transgenic data presented in this study allow an interpretation of the adult En-2 mutant cerebellar phenotype. In the mutant En-2 cerebellar hemispheres, three instead of four lobes were present due to the failure in formation of the ansiparamedial fissure. In the posterior vermis, there were disruptions in the En-2 mutant cerebellum around a fissure, designated 2/py, that could not be defined unambiguously as the prepyramidal or the secondary fissure. This resulted in a reduction in the size of the adjacent VIIIth lobe and its association with the IXth instead of the VIth and VIIth lobes. Finally, altered lacZ staining patterns in transgenic mice that were homozygous for the En-2<sup>nh2</sup> mutation suggested changes in lobe identity in the paraflocculus and the VIIth and VIIIth posterior vermal lobes, which may account for the large reduction in size of these lobes.

Both the exon 1 and exon 2 deletion mutants of En-2 appear to produce the same phenotype of defective cerebellar patterning. The En-2<sup>ndh2</sup> allele is predicted to be a null allele, since the DNA sequences coding for the first 108 amino acids are deleted and no homeodomain-containing protein could be detected in En-2<sup>ndh2</sup> homozygous mutants. Together, these findings suggest that both En-2 mutations are null alleles. This is in contrast to results from gene targeting experiments of hox-a-1 (Lufkin et al., 1991, Chisaka et al., 1992) and hoxb-4 (Ramirez-Solis et al., 1993), where exon 1 and exon 2 mutant alleles of the same locus have been shown to result in different mutant phenotypes. This suggests that the underling biochemical consequences of individual mutations can not be easily predicted and each mutation must be analyzed on an individual basis.

During normal fissure formation, once fissures are initiated they remain and become progressively more defined. An unexpected feature of the En-2 phenotype was the establishment, disappearance and reappearance of the 2/py fissure during P5 through P7 of cerebellar development. The underlying cellular mechanism responsible for this remains unclear. During postnatal development, the EGL is the only mitotically active cell type in the cerebellum and it has been suggested that differential proliferation of this layer is the driving force behind cerebellar foliation (Mares et al., 1970; Mares and Lodin 1970). The disappearance of the 2/py fissure could be explained by massive cell death of the EGL in the region between P5 and P6. A second round of localized proliferation of the remaining EGL cells could then be responsible for the return of the fissure. However, we saw no histological indications of large numbers of apoptotic or necrotic cells in the EGL at any stage in En-2 mutants (data not shown). Alternatively, cell migration or differential rates of proliferation may account for this observation. These alternatives could be distinguished by a careful study of proliferation patterns in the mutant and wild-type EGL.

Lethal mutations in the *Drosophila en* gene result in segment fusions, whereas a viable *en* mutation results in a posterior to anterior wing transformation (Lawrence and Morata, 1976; Nusslein-Volhard and Wiedmaier, 1980 and Kornberg, 1981). Our morphological analysis of mouse En-2 mutants has
uncovered abnormal cerebellar fissure formation resulting in lobe fusions and our analysis of transgene expression suggests that changes in cerebellar lobe identities have occurred. Two of the changes in lacZ expression suggest posterior to anterior transformations; posterior vermal lobe VI to anterior lobe VI and posterior paraflocculus to anterior paraflocculus. The third example suggests an anterior to posterior lobe transformation; vermal lobe VIII to lobe IX, which was further supported by the developmental profile data. Several observations support the contention that these are indeed lobe transformations and not simply due to insignificant changes in lacZ gene expression or Purkinje cell loss. Firstly, the β2γ pattern of lacZ-positive and -negative cells defines entire lobes of the cerebellum. In the mutants, the staining patterns of whole lobes are affected, not just individual cells; a loss of lacZ expression in some cells and a gain of expression in others. Secondly, the posterior to anterior lobe VI transformation was observed with two separate transgenes that mark two different cell types. LacZ expression is restricted to a subset of Purkinje cells in the β2γ transgenic line whereas granular cells express the Tg5 transgene. Finally, because En-2 is not expressed in adult Purkinje cells, the change in pcp2 lacZ expression in adult Purkinje cells in En-2<sup>2nd</sup> homozygous mutant animals cannot be directly due to the lack of En-2 protein in Purkinje cells. This suggests that the En-2<sup>2nd</sup> mutation is altering the identities of Purkinje cells. Furthermore, the altered lacZ expression pattern in β2γEn-2<sup>2nd</sup> compound mutants was observed as early as 4 days after birth when the transgene is first expressed (data not shown), suggesting that the Purkinje cell identities were altered at an early stage of cerebellar development.

An En-2 embryonic cerebellar phenotype was clear by 15.5 d.p.c. as a decrease in the extent of fusion of the cerebellar rudiments. This occurs at least 2 days prior to the initiation of foliation. This suggests that the initial defect in En-2 mutant cerebellar development may not be the postnatal process of foliation itself, but the embryonic process of generating cerebellar precursors. This may be due to either a delay in production of the cerebellar neurons or a deletion of some of these cells. The latter possibility is supported by the smaller size of the adult cerebellum (Joyner et al., 1991). However, size alone does not seem to be a critical factor in determining the patterning of cerebellar foliation. For example, undernourishment of rats during development results in smaller, but correctly patterned cerebellar folia (Vitiello and Gombos, 1987).

At 15.5 d.p.c., when a phenotype can be seen, the bulk of the cerebellum is made up of Purkinje, Golgi and deep nuclei cells. Since our previous studies have shown that En-2 expression in the cerebellum is widespread at embryonic stages (Davis and Joyner, 1988; Davis et al., 1988), any or all of these cell types could be the primary cell(s) affected in En-2 mutant cerebella. Although the dramatic increase in granule cell number is thought to cause the formation of the folds, there is little evidence implicating a specific cell type in controlling the pattern of cerebellar foliation. However, Purkinje cells, which have been shown to be important in patterning of cerebellar afferents (Arsenio-Nunes et al., 1988), do at least seem to be required for cerebellar foliation. When subpopulations of Purkinje cells are ablated at birth by SV40 T-antigen expression in transgenic mice, granule cell proliferation and foliation is arrested in the regions of cell death (Fedderden et al., 1992). Further evidence from both reeler (Mallet et al., 1977) and staggerer mutant mice (Herrup and Mullen, 1979) suggests that the rate of granule cell division in the EGL may be controlled by the nearby Purkinje cells.

An important question raised by our studies is whether the embryonic cerebellar phenotype is related to the postnatal foliation patterning defect. Recent expression studies (K. Millen, C. c.–Hui and A. Joyner, unpublished data) have shown that En-2 normally is not expressed in the cerebellar hemispheres at P6 when abnormal fissure formation occurs in the mutant hemispheres. However, En-2 is expressed across the developing wild-type cerebellum, transiently between 15.5 to 17.5 d.p.c., with an interesting pattern of sagittal stripes of higher expression. These stripes are reminiscent of sagittal stripes that have been seen with some Purkinje cell antigens. Wassef et al. (1992) have speculated that these markers are a reflection of a combinatorial topographic map of the cerebellum that gives Purkinje cells spatially distinct molecular labels that are used to establish a cerebellar projection map (reviewed by Wassef et al., 1992). Therefore, En-2 might play a role in the spatial patterning of the cerebellum during embryogenesis.

Our expression analysis, combined with the phenotype analysis are consistent with the embryonic phenotype being directly related to the postnatal foliation patterning defect. If this is the case, then one would predict that either a specific set of pre-patterned cells is deleted in En-2 mutants or that a normal number of embryonic cells are generated, but either their identities or birth dates are altered, leading to a loss of Purkinje cells and a decrease in granule cell number. It is interesting to speculate that the normal spatially restricted expression of En-2 and other genes in the embryonic cerebellum may generate spatial cues used in the patterning of cerebellar foliation as well as the patterning of cerebellar afferents. Further studies are necessary to determine what cells are the primary cell type(s) affected in En-2 mutants. A detailed analysis of the proliferation patterns and numbers of Purkinje and other cells in En-2 mutants, as well as a detailed analysis of the expression of genes expressed in sagittal stripes during embryogenesis in En-2 mutant animals should provide further insight into the cellular basis of the En-2 cerebellar patterning defect during embryogenesis.

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