Genetic subdivision of the tectum and cerebellum into functionally related regions based on differential sensitivity to engrailed proteins

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The genetic pathways that partition the developing nervous system into functional systems are largely unknown. The engrailed (En) homeobox transcription factors are candidate regulators of this process in the dorsal midbrain (tectum) and anterior hindbrain (cerebellum). En1 mutants lack most of the tectum and cerebellum and die at birth, whereas En2 mutants are viable with a smaller cerebellum and foliation defects. Our previous studies indicated that the difference in phenotypes is due to the earlier expression of En1 as compared with En2, rather than differences in protein function, since knock-in mice expressing En2 in place of En1 have a normal brain. Here, we uncovered a wider spectrum of functions for the En genes by generating a series of En mutant mice. First, using a conditional allele we demonstrate that En1 is required for cerebellum development only before embryonic day 9, but plays a sustained role in forming the tectum. Second, by removing the endogenous En2 gene in the background of En1 knock-in alleles, we show that Drosophila en is not sufficient to sustain midbrain and cerebellum development in the absence of En2, whereas En2 is more potent than En1 in cerebellum development. Third, based on a differential sensitivity to the dose of En1/2, our studies reveal a genetic subdivision of the tectum into its two functional systems and the medial cerebellum into four regions that have distinct circuitry and molecular coding. Our study suggests that an ‘engrailed code’ is integral to partitioning the tectum and cerebellum into functional domains.

KEY WORDS: Cerebellum, Foliation, Tectum, Patterning, En1, En2, Mouse

INTRODUCTION

The genetic regulation of patterning processes that regulate the size, cellular differentiation and morphology of regions of the developing nervous system is fundamental to establishing functional circuits that control different behaviors, emotions and basic bodily functions. The embryonic brain region that gives rise to the midbrain [mesencephalon (mes)] and anterior hindbrain [rhombomere (r1)] is an ideal model system for studying these genetic pathways in vertebrates. Anterior-posterior (A-P) patterning of the midbrain (Mb) and anterior hindbrain is orchestrated by an organizing center in the isthmus located between the mes and r1. Fgf8 is the key isthmic organizer molecule that acts between embryonic day (E) 8.5 and 13 to regulate the expression of genes that direct Mb and r1 development (Wurst and Bally-Cuif, 2001; Zervas et al., 2005). Expression of the engrailed transcription factors (En1 and En2) before E13 is regulated by Fgf8, and En1/2 are crucial for mes/r1 development. It has been challenging to determine the full spectrum of functions of each En gene in mouse because there is an early loss of the mes/r1 in En1 mutants, and the two genes have overlapping functions. It is also unclear whether the two En proteins have equivalent functions in brain development.

Following specification of the mes/r1 region during neural tube closure, the mouse tectum and cerebellum (Cb) develop from the dorsal mes and r1, respectively (Zervas et al., 2005). The tectum of the Mb forms as a layered structure that is divided morphologically and functionally into the anterior superior colliculus and posterior inferior colliculus that process visual and auditory information, respectively. Although expansion of the tectum along the A-P axis is tightly linked to the level of isthmic organizer signaling, the molecular basis of differential allocation of the inferior and superior colliculi is not understood. The Cb is the center for motor control. Differentiated cells of the mouse Cb begin to be generated at E10.5 and form a multi-laminar structure consisting of the deep nuclei surrounded by a dense layer of granule cells, a monolayer of Purkinje cells and an outer molecular layer. The granule cell precursors form a proliferative external granule layer at E13.5 and then migrate past the Purkinje cell layer to form the inner granule layer (IGL) from birth until postnatal day (P) 14. Beginning at E17.5, fissures form in a stereotyped manner and generate a highly foliated Cb. In terms of how early A-P patterning could influence the final structure of the Cb, it is important to note that a morphogenetic rotation of dorsal r1 transforms the A-P axis of r1 into the medial-lateral (M-L) axis of the Cb primordium by E12.5 (Sgaier et al., 2005) (see Fig. 8). Globally, the adult Cb is subdivided into a medial vermis and two lateral hemispheres, with the vermis divided along the A-P axis by 8-10 folia in different inbred mouse strains (referred to as I-X) and the hemispheres divided by 6 folia (Larsell, 1952). Preservation of the general pattern of folia across mammals suggests that there is an evolutionarily conserved genetic program that patterns folia of the Cb (Altman and Bayer, 1997; Herrup and Kuemerle, 1997).

The mouse En1 and En2 genes provide a unique tool for gaining access to the genetic regulation of Cb and tectum patterning. The dynamic expression patterns of the En genes (see Fig. 1) and their
mutant phenotypes reflect each successive stage of Cb and tectum development (Joyner, 1996). En1 is first expressed in the mes/r1 at E8.5, ~12 hours before En2, and is later expressed in the absence of En2 in a number of other tissues. En1-null mutant mice die at birth and have an almost complete deletion of the Mb and Cb owing to tissue loss by E9.5 (Wurst et al., 1994), which is caused, at least in part, by cell death (Chi et al., 2003). Thus, En1 is required for the initial establishment of the mes/r1 region. By contrast, En2-null mutants have a mild phenotype – they are viable and have defects limited to growth of the Cb and patterning of particular folia (Joyner et al., 1991; Millen et al., 1994). An overlap in En gene function has been demonstrated by the complete absence of the tectum and Cb in En1;En2 double mutants (Liu and Joyner, 2001; Simon et al., 2004), and a rescue of the En1 mutant brain phenotype when En1 is replaced with En2 using gene targeting (Hanks et al., 1995). Surprisingly, we found that Drosophila en also can rescue the En1 mutant brain defects in knock-in mouse mutants, although en cannot rescue other defects (Hanks et al., 1998). An important question is whether En1 has any later roles in tectum and Cb patterning, as has been suggested by the Cb phenotype of En1-null mutants that survive on a C57BL/6 genetic background (Bilovocky et al., 2003), and the degree to which such functions overlap with En2.

In order to study the temporal requirement for En1 in mes/r1 development, we generated a conditional mutant allele of En1. We find that if En1 is removed at ~E9, only the posterior tectum is depleted, and two copies of En2 are required to sustain Cb development in these conditional En1 mutants. We next compared the function of Drosophila and mouse En proteins in the mouse brain using a sensitive genetic assay. We provide evidence that En1 is more potent at supporting Cb development than En2, and En2 cannot rescue the En1 mutant brain defects in the absence of endogenous En2. Curiously, our analysis of knock-in mutants and En1/2 double-null mutants uncovered that both genes are preferentially required in particular functional domains of the tectum and cerebellum. We propose an ‘En code’ that divides the tectum and Cb into functional systems based on the dose of En required for the development of each domain.

**MATERIALS AND METHODS**

**Generation of En1flank** knock-in mice and conditional ablation of En1 in r1

The En1flank targeting construct was produced by subcloning a 6.0 kb S′ BamHI En1 sequence into the BamHI site of pPNTfnt-Neot-flox-loxP to generate pPNTfnt-Neot-floxP+En1-5′ arm. A 2.7 kb BamHI-Xbal fragment of En1 including most of exon 2 was subcloned into the Xbal site of the pGEM-11ZF vector to generate pGEM-11ZF+En1-3′ arm. A loxP sequence was inserted into the KpnI site within the 3′ UTR of En1 and the En1-loxP-3′ arm was released by Xbal and Sall digestion and subcloned into the HindIII site of pPNTfnt-Neot-floxP+En1-5′ arm to generate the final targeting vector. The En1flank targeting construct was linearized with Sall and electroporated into W4 embryonic stem (ES) cells (Auerbach et al., 2000) as described previously (Matise et al., 2000). Clones were screened by Southern blot analysis using 5′ external and 3′ internal probes to identify targeted clones (see Fig. S1 in the supplementary material). One positive clone was obtained and injected into C57BL/6 blastocysts to generate ES cell chimeric mice (Papaioannou and Johnson, 2000). Chimeric mice were mated with Black Swiss mice to generate En1loxPflank mice. The neo cassette was removed by mating En1loxPflank mice with MGTB-Fpβ mice (Rodriguez et al., 2000), which expresses Fpβ broadly under the control of the human β-actin promoter. The wild-type (337 bp) and En1flank (380 bp) alleles were detected by PCR with the following primers: En1lox1A, 5′-GCCAAACTGCTTACGACCCG-3′; En1lox1B, 5′-TGGGTGGTTAGAGAGGAGG-3′.

Mes/r1-specific En1 conditional mutant mice (En1flank) were generated by crossing En1flank mice with En1Cre+ mice. En1Cre+ and En1Cre+En1Cre+ mice were generated within the same litter by crossing En1flank mice with En1Cre+ mice. En1Cre+:En2flox/– were bred to En2flox/– mice to generate En1Cre+:En2flox/– and En1Cre+:En2flox/– mice, which are caused, at least in part, by cell death (Chi et al., 2003). Thus, En1 is required for the initial establishment of the mes/r1 region. By contrast, En2-null mutants have a mild phenotype – they are viable and have defects limited to growth of the Cb and patterning of particular folia (Joyner et al., 1991; Millen et al., 1994). An overlap in En gene function has been demonstrated by the complete absence of the tectum and Cb in En1;En2 double mutants (Liu and Joyner, 2001; Simon et al., 2004), and a rescue of the En1 mutant brain phenotype when En1 is replaced with En2 using gene targeting (Hanks et al., 1995). Surprisingly, we found that Drosophila en also can rescue the En1 mutant brain defects in knock-in mouse mutants, although en cannot rescue other defects (Hanks et al., 1998). An important question is whether En1 has any later roles in tectum and Cb patterning, as has been suggested by the Cb phenotype of En1-null mutants that survive on a C57BL/6 genetic background (Bilovocky et al., 2003), and the degree to which such functions overlap with En2.

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**RESULTS**

**En1 is not required after ~E9.0 for development of the Cb and superior colliculus**

Our finding that En2 can fully rescue the En1 mutant phenotype in En1En2 knockout mice (Hanks et al., 1995) indicates that the two En proteins are functionally interchangeable and that the brain defects in En1 and En2 single mutants arise from cells that express only one En gene at critical time points in development. To address the question of whether En1 can compensate for En1 after the mes/r1 is specified, we first delineated the dynamic expression patterns of the En genes using En1lacZ (Hanks et al., 1995; Matise and Joyner, 1997) and En2in-lacZ (Sgaier et al., 2005) knock-in alleles. En1lacZ expression was first detected at the two-somite stage broadly spanning the mes/r1 region (Fig. 1A) and then progressively narrowed around the mes/r1 junction.
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(isthmic organizer) (Fig. 1B-D). En2-tau-lacZ expression commenced at the five-somite stage in a subdomain of the En1-positive region (Fig. 1G) and quickly broadened (Fig. 1G-J). Within the bilateral wing-like structure of the E12.5 cerebellar primordium (CbP), En1-lacZ was restricted to the medial-most region (vermis anlage), whereas En2-tau-lacZ was expressed in all but the most-lateral CbP (Fig. 1E,K). Similarly, in the Mb, En2-tau-lacZ expression extended more rostrally than En1-lacZ (into the anlage of the superior colliculus). Furthermore, expression of both En genes formed a mirror image double gradient across the mes/r1 region, with highest levels in the isthmus. Starting at E15.5, En1 and En2 expression changed to a sagittal striped pattern along the M-L axis of the CbP, owing to regional downregulation (Fig. 1FL) (Millen et al., 1995). Thus, the En genes have dynamic gene expression patterns, but after ~E9 (including postnatal stages; data not shown) the En1 expression domain appears to be encompassed within the En2 expression domain.

The expression analysis of En1 and En2 suggests that En1 might only be required in the mes/r1 before ~E9. To determine whether this is the case, we generated a conditional mutant allele of En1 (En1flox/+;En2–/+) in which the coding sequences in the second exon are flanked with loxP sites (see Fig. S1 in the supplementary material). Germline chimeras were bred to Black Swiss mice to avoid rescue of the En1 mutant phenotype by the C57BL/6 background (Bilovocky et al., 2003). In order to delete En1 at ~E9.0, we combined the En1flox allele with the null En1Cre knock-in allele (Kimmel et al., 2000). Within the mes/r1 region, Cre function is first detected at the five-somite stage and is active in all mes/r1 cells by 15 somites (E9.0) (Li et al., 2002). In En1floxCre mice, the second exon of the En1flox allele thus begins to be deleted shortly after its expression is induced, and En1 is expressed for only approximately 24 hours (Fig. 2A).

Indeed, En1floxCre mice were found to be viable and survive to adulthood. En1floxCre mutants had a limb phenotype similar to the rare En1 mutants that survive (Loomis et al., 1996) (data not shown). The brains of all but one adult En1floxCre mouse analyzed (n=8) appeared grossly normal in whole-mount (Fig. 2B,C). The one mouse that was different had a partial deletion of the Mb and Cb (data not shown). Analysis of sagittal sections of the remaining En1floxCre mice revealed that the inferior colliculus (posterior tectum) was partially truncated (in all eight) (Fig. 2E,F and Table 1; compare also with Fig. 3B,I). In addition, five of the seven En1floxCre mice had a mild foliation defect in the anterior vermis (medial Cb), and the overall size of the vermis was slightly smaller than normal. The fissure between the anterior-most folia (I/II and III) either failed to form (in two of five) (Fig. 2F), or was shallower than normal (in three of five) in these mutants. Of significance, in two of the seven mutants analyzed, the fissure between folia II and III appeared as deep as in wild-type brains (Fig. 2D,E). One likely possibility for the variable rescue in the Cb is that in the two En1floxCre mutants that had a normal Cb, En1 was ablated at a slightly later stage. Interestingly, in all eight of the En1floxCre mice analyzed, the superior colliculus and the hemispheres (lateral Cb) appeared normal (Fig. 2D-I). Thus, our analysis of the requirement for En1 after ~E9 demonstrates that two copies of En2 are sufficient to support Cb development, despite being expressed in a broader domain of the tectum than En1, En2 alone is not able to fully regulate inferior colliculus development.

One copy of En2 is not sufficient to support development of the remaining inferior colliculus or the Cb when En1 is deleted by E9

To determine whether one copy of En2 is sufficient to support development of the superior colliculus and Cb when En1 is removed at ~E9, we removed one copy of En2 on the En1floxCre background. Strikingly, En1floxCre;En2+/- mutant mice were not found at weaning (42 mice were analyzed from five litters of a cross between En1flox/+;En2+/- and En1flox+/-;En2+/- mice). We therefore analyzed the phenotypes of En1floxCre embryos lacking one En2 allele at E18.5, and compared them with En1+/- and En1–/–;En2+/- double mutants (Fig. 3). As expected, at E18.5, the vermis of En1floxCre;En2+/- mice was either normal or slightly delayed in forming folia (Fig. 3B,I and Table 1). By contrast, En1floxCre;En2+/- mice (Fig. 3J) displayed a complete deletion of the Cb that was very similar to En1+/-;En2+/-
(Fig. 3G) and En1<sup>−/−</sup>:En2<sup>−/+</sup> (Fig. 3H) mutant embryos and more severe than En1<sup>−/−</sup>:En2<sup>−/−</sup> mutants (Fig. 3F) that had some lateral Cb tissue remaining (see also Table 1). However, unlike En1<sup>−/−</sup>:En2<sup>−/−</sup> (Fig. 3G) and En1<sup>−/−</sup>:En2<sup>−/+</sup> mutants (Fig. 3H), which have no tectum, in En1<sup>flox/Cre</sup>;En2<sup>−/+</sup> mutant mice (Fig. 3J) some superior colliculus tissue remained, similar to the phenotype of En1<sup>−/−</sup>:En2<sup>−/−</sup> mice (Fig. 3F and Table 1). By contrast, En1<sup>flox/Cre</sup>;En2<sup>−/+</sup> mutants had a complete deletion of the Cb and tectum (Fig. 3K and Table 1).

In summary, when En1 is expressed until ~E9.0 (En1<sup>flox/Cre</sup>), two copies of En2 can support superior colliculus and Cb development.

### Table 1. Summary of En1/2 and En1 conditional mutant phenotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>En1 alleles</th>
<th>En2 alleles</th>
<th>SC</th>
<th>IC</th>
<th>Cb size</th>
<th>aV (I-V)</th>
<th>pH (CII/PM)</th>
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<td>Denki/Denki</td>
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<td>flox/Cre</td>
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<td>Denki+/+</td>
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En1/2 alleles: +, wild type; −/−, null. SC/IC: ++, normal superior/inferior colliculus; X, reduced in size; X, little formed; X, not formed. Cb size: ++, normal; X, ~1/3 overall reduction; X, ~1/2 overall reduction; ( ), only the most lateral tissue remains; X, not formed. Anterior vermis (aV): ++, normal folia I-V; X, fusion and smaller folia I-V; ++, fusion and smaller folia I-V. Posterior vermis (pV): ++, folium VIII is associated normally with folium VII; X, folium VIII positioned between lobule VII and IX; X, folium VIII is smaller and associated with folium IX; X, little or no folium VIII. Posterior hemisphere (pH): ++, crus and paramedian folia are normal; X, crus and paramedian folia are fused; X, one mutant had a partial fissure separating crus and paramedian. Ψ, denotes the mutants that survived into adulthood and that were analyzed.
and partial development of the inferior colliculus, whereas one copy of \( En2 \) is not sufficient to support any development of the inferior colliculus or Cb, and in the absence of all \( En2 \) the superior colliculus also does not form.

**Particular regions of the Mb and Cb are sensitive to the dose of \( En1 \) and \( En2 \)**

Since our analysis of \( En1 \) conditional mutants showed that \( En1 \) is preferentially required in the inferior colliculus and folia I-III, we further explored the requirement for each mouse En gene in mes/r1 development by analyzing viable \( En1 \) and \( En2 \) double mutants. Surprisingly, although the Cb of \( En1^{+/+} \) or \( En2^{+/+} \) mice on an outbred background is normal (data not shown), section analysis of \( En1^{+/+};En2^{+/+} \) double heterozygotes (Fig. 4A,E,C,G) revealed two foliation defects in the vermis. One was a variable but consistent (\( n=7 \) of 8) partial fusion of the three anterior-most folia (I-III), similar to the phenotype seen in some \( En1^{\text{floox/Cre}} \) mutants (Fig. 2F and see Table 1). \( En1^{+/+};En2^{+/+} \) mice (Fig. 4G) had an additional slight posterior shift in the position of folium VIII that was milder than in \( En2^{-/-} \) mutants (Fig. 4F), in which folium VIII is associated

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**Fig. 3. Differential requirement of En genes for mouse midbrain and cerebellar development.**

(A) Schematic illustrating the detailed expression profile of each En allele \((En1\), \( En2 \) and \( En1^{\text{floox/Cre}} \)) within the mes/r1 region. (B-K) Cresyl Violet-stained mid-sagittal sections of E18.5 brains with the corresponding genotypes and all the functional En allele profiles indicated at the top. Asterisks indicate forming fissures. Black arrows indicate truncated inferior colliculus. The inset in F shows the lateral Cb tissue that forms in the \( En1^{-/-} \) mutants.
with folium IX instead of with VI/VII. Furthermore, there was a general delay in fissure formation in the vermis of E18.5 En1<sup>−/−;En2<sup>−/−</sup> mutants (Fig. 3B,C), similar to but milder than the delay in En2<sup>−/−</sup> mutants (Fig. 3D) (Millen et al., 1994).

En1<sup>−/−;En2<sup>−/−</sup> mice, which have a smaller Mb and Cb than normal at E18.5 (Simon et al., 2005), were found to survive to adulthood. En1<sup>−/−;En2<sup>−/−</sup> mutants (Fig. 3B,E) had a more severe delay in Cb foliation than En2<sup>−/−</sup> mutants (Fig. 3D). Consistent with this, the Cb of adult En1<sup>−/−;En2<sup>−/−</sup> mutants (Fig. 4D,H,L) had a variable but greater size reduction and more foliation defects than En2<sup>−/−</sup> (Fig. 4B,F,J) or En1<sup>−/−;En2<sup>+/−</sup> mice (Fig. 4C,G,K and see Table 1). In the majority of the mutants (six out of eight) the five anterior-most folia (I-V) of the vermis were replaced by a single fold. In addition, folium VIII was more defective than in En2<sup>−/−</sup> mutants: it was absent or decreased in size and misaligned with folium IX. The inferior colliculus also was partially truncated. In one of the mutants, folia I-V were almost completely lost and fused to a more profoundly truncated tectum (data not shown). Interestingly, in all the viable En1<sub>2</sub> mutants the relative thickness of the remaining molecular and granule cell layers and Purkinje cell density were normal. Our analysis of viable En1<sub>2</sub> mutants provides additional evidence that the anterior vermis (folia I-V) and inferior colliculus are most sensitive to a reduction in En1, and also reveals redundant functions for En1 and En2 in folia I-V and VIII.

**En2 can sustain more extensive Cb vermis development than En1**

In order to directly compare the in vivo functions of En1 and En2 proteins, we took advantage of our En1<sub>En2ki</sub> knock-in mice (Hanks et al., 1995). A sensitive assay for comparing protein function is to combine knock-in alleles with loss-of-function alleles (Wang and Jaenisch, 1997). If the two En proteins have equivalent properties, then removal of En2 in En1<sub>En2ki/En2ki</sub> mice (En1<sub>En2ki/En2ki;En2<sup>−/−</sup></sub>) should only cause the foliation defects seen in En2<sup>−/−</sup> mice. Indeed, when expressed in two copies from the En1 locus, En2 is sufficient to support development of the inferior colliculus. Unlike En1<sub>En2ki/En2ki;En2<sup>−/−</sup></sub> mice, which have a partial truncation of the tectum, the inferior colliculus appeared normal in En1<sub>En2ki/En2ki;En2<sup>−/−</sup></sub> adult or early postnatal mice (Fig. 5A,C and data not shown). Furthermore, consistent with En1 expression being restricted to the primordium of the vermis, as compared with the broader En2 expression in the hemispheres after ~E9.5, the hemispheres of En1<sub>En2ki/En2ki;En2<sup>−/−</sup></sub> mice (Fig. 5G,I) had the characteristic En2<sup>−/−</sup> phenotype (Fig. 4J). However, unlike En2<sup>−/−</sup> mice (Fig. 4F), in which folium VIII is associated with folium IX, in three out of four adult En1<sub>En2ki/En2ki;En2<sup>−/−</sup></sub> mice (Fig. 5A,C), folium VIII was normal. This shows that the vermis foliation defects in En2<sup>−/−</sup> mice can be rescued by expressing the En2 gene, but not the En1 gene, from the En1 locus. This suggests that En1 and En2 proteins are not equivalent, but rather that En2 activity is either specifically required in the posterior Cb (folium VIII) or more active in the Cb.

We further tested whether En2 is generally more potent than En1 by comparing the phenotype of En1<sup>−/−;En2<sup>−/−</sup></sub> mice with En1<sub>En2ki/−</sub>;En2<sup>−/−</sup> mice. Strikingly, we found that En1<sub>En2ki/−</sub>;En2<sup>−/−</sup> mice (n=3) had a much milder anterior Cb phenotype than En1<sup>−/−;En2<sup>−/−</sup></sub> mutants. The anterior vermis of En1<sub>En2ki/−</sub>;En2<sup>−/−</sup> mutants (Fig. 5A,D) had three distinct folia (I/II, III and IV/V) compared with one in En1<sup>−/−;En2<sup>−/−</sup></sub> mice (Fig. 4H). In addition, in the posterior vermis of En1<sub>En2ki/−</sub>;En2<sup>−/−</sup> mice (Fig. 5D), folium VIII was only partially displaced toward folium IX, in contrast to En1<sup>−/−;En2<sup>−/−</sup></sub> mutants (Fig. 4H), which have a substantial deletion of folium VIII. This sensitive dosage assay of one copy of En2 or En1 expressed from the En1 locus in the absence of all other En function thus indicates that En2 is generally more active than En1 in regulating Cb development. Finally, the posterior tectum of En1<sub>En2ki/−</sub>;En2<sup>−/−</sup> mice (Fig. 5D) was partially truncated (Fig. 4H and data not shown), seemingly less than in En1<sup>−/−;En2<sup>−/−</sup></sub> mutants.


**Drosophila en expressed in place of En1 cannot support mes/r1 development in the absence of En2**

Our finding that one or two copies of En2 expressed from the En1 locus in the absence of endogenous En2 can support tectum and Cb development prompted us to determine whether Drosophila en could do the same. To determine whether *Drosophila en*, when expressed in place of En1, can sustain mes/r1 development in the absence of En2, we made use of knock-in mice (*En1Denki*) that express *Drosophila en* in place of *En1* (Hanks et al., 1998). Whereas *En1* and *En2* mice are viable, we did not detect any *En1Denki;En2* mice after birth. Section analysis of E18.5 *En1Denki;En2* embryos revealed either the same phenotype as *En1*;*En2* embryos (n=1 of 3) or a similar phenotype to *En1floxCre;En2* mutants (n=2 of 3; data not shown).

Interestingly, half the expected number of *En1Denki;En2* mutants survived to adulthood, indicating that *Drosophila en* has some En1-like activity as *En1*;*En2* die at birth. Histological analysis of the brains of three *En1Denki;En2* mice (Fig. 5A,E and Table 1) that survived surprisingly revealed that two of the mutants had a normal tectum and Cb, whereas one had a partial deletion of the posterior tectum and a vermis foliation defect similar to *En1*;*En2* mutants (Fig. 4H). The five *En1Denki;En2* mice analyzed had a phenotype that was milder than *En1*;*En2* mice, with a distinct IV/V folium (Fig. 5A,F and Fig. 4H). All but one of the five *En1Denki;En2* mutants analyzed had an *En2* foliation pattern in the hemispheres (Fig. 4J, Fig. 5G.L). These results indicate that *Drosophila en* function can partially replace normal En1 function in the initiation of normal En2 expression (before ~E9.0) in *En1Denki;En2* mice, after which mouse En2 contributes a necessary function for continued normal development of mes/r1-derived structures.

**Medial-lateral patterning of the Cb appears to be altered in En1*+/+;En2*−/− mutants**

One possible reason for the loss of the posterior tectum and anterior vermis in *En1*;*En2* adult mutants is specific loss of the cells that give rise to these two regions (caudal mes and rostral r1) (Sgaier et al., 2005; Zervas et al., 2004). To determine whether this is the case, we fate mapped the posterior mes and medial r1 in *En1*;*En2* mutants by genetic inducible fate mapping (GIFM) (Joyer and Zervas, 2006) using our null *En1CreERT1*/(−/−) allele (Sgaier et al., 2005) and the R26R lacZ reporter allele (Soriano, 1999). When tamoxifen (TM) is administered at 18.00 h to E10.5 wild-type (*En1CreERT1*/+;*En2*+/+;R26R+) embryos (marking cells at E11-12), the posterior mes and medial-most domain of the E12.5 CbP are marked (Fig. 6A) and give rise to the vermis and inferior colliculus (Fig. 6C). At E16.5, the size of the marked population of cells in the Cb appeared wider in *En1CreERT1*/−;*En2*−/−;R26R+ embryos (Fig. 6D) than in wild types (Fig. 6C). By contrast, the size of the marked domain in the tectum was smaller than in wild types (Fig. 6C-F). Similarly, in adult *En1CreERT1*/−;*En2*−/−;R26R+ mutants, the marked domain in the Cb was broader than normal, whereas the size of the marked domain in the tectum was greatly reduced compared with wild types as it was restricted to the remaining inferior colliculus (Fig. 6G-J). The fate mapping results in the mes suggest that En1 function can partially replace normal En2 function in the initiation of normal En2 expression (before ~E9.0) in *En1Denki;En2* mice, after which mouse En2 contributes a necessary function for continued normal development of mes/r1-derived structures.
that in En1/2 mutants, the posterior mes cells marked at E12.5 do not expand normally and this results in a smaller inferior colliculus in the adult. By contrast, because the domain of marked cells in the adult Cb is larger than normal even though the vermis is reduced in size, this suggests that the anterior r1 cells marked at E12.5 are not only retained but contribute to more lateral regions of the vermis than normal. Thus, the loss of tectum and vermis tissue in En1<sup>-/-</sup>:En2<sup>-/-</sup> adult mutants is not simply owing to loss of the cells that give rise to the these two regions.

The isthmic organizer is not lost in En2<sup>+/+</sup> and En1<sup>-/-</sup>;En2<sup>-/-</sup> mutants

Since Fgf8 expression is lost in En1/En2 double-homozygous mutant embryos by E9 (Liu and Joyner, 2001), one possible reason for the defects in En2<sup>-/-</sup> and En1<sup>-/-</sup>;En2<sup>-/-</sup> mutants is a disruption of the isthmic organizer (decrease in Fgf signaling). However, our previous whole-mount RNA in situ analysis of En1<sup>-/-</sup>;En2<sup>-/-</sup> embryos from the six-somite stage to E9.5 did not reveal any obvious changes in the expression of Fgf8 or mes/r1 morphology (Liu and Joyner, 2001). Based on section analysis, En2<sup>-/-</sup> embryos had a slight reduction in the size of the Cb by E11.5, and En1<sup>-/-</sup>;En2<sup>-/-</sup> mutants had a greater reduction in the CbP and truncation of the tectum (see Fig. 7). We therefore performed RNA in situ analysis at E11.5, a day before Fgf8 expression is normally terminated in the isthmus. Consistent with the fate mapping study, we found that the expression domain of En1 was not obviously altered along the A-P axis in the mutants (Fig. 7G-I). The only obvious difference in the expression domains of Fgf8 and Fgf17 (a related organizer gene) and of the direct target gene Spry1 (Liu et al., 2003) between En2<sup>-/-</sup> and En1<sup>-/-</sup>;En2<sup>-/-</sup> mutants as compared with wild-type mice (En1<sup>-/-</sup>) was a slight reduction in the size of the domains, which correlated with the reduction in mes/r1 tissue in each mutant (Fig. 7A-F and data not shown). Consistent with retention of organizer activity, the expression domains of two mes genes (Otx2 and Wnt1) and one r1 gene (Gbx2) regulated by Fgf8, were not altered (data not shown).

**DISCUSSION**

In this study, we have analyzed a series of mouse En conditional knock-in and null mutants to decipher the overlapping and individual functions of the two highly conserved En genes in mes/r1 development. Overall, we found that the inferior colliculus of the tectum and three regions of the Cb are particularly sensitive to the level of En genes (see below and Table 1). Interestingly, the anterior vermis and tectum defects we observed in En1<sup>-/-</sup>En2<sup>-/-</sup>, En1<sup>-</sup>Denki<sup>-/-</sup>;En2<sup>-/-</sup> and En1<sup>-/-</sup>;En2<sup>-/-</sup> mutant mice have similarities to Fgf8<sup>+/+</sup>;Fgf17<sup>+/+</sup> mutants (Xu et al., 2000), raising the possibility that a key role of En1/2 is to maintain Fgf8 expression (Liu et al., 2003). We found that Fgf8 expression is maintained as long as one allele of En1 is present, although there are subtle decreases in Fgf8/17 expression. Since En1/2 expression persists after E12.5, when Fgf8 expression is terminated, and En1<sup>-/-</sup>;En2<sup>-/-</sup> mutants have a much more severe loss of the tectum and Cb folia than Fgf8<sup>+/+</sup>;Fgf17<sup>+/+</sup> mutants, it is possible that En1/2 do not control mes/r1 development solely through regulating Fgf8/17 expression.

**En1/2 differentially regulate retention of cells in the mes and r1**

By determining the fate of the En1-expressing cells at ~E11, which normally give rise to the vermis and inferior colliculus, in En1<sup>-/-</sup>;En2<sup>-/-</sup> mutants using GIFM, we uncovered an unexpected differential role for En1/2 in regulating growth and survival of cells in the tectum versus the Cb. In En1/2 mutants, the posterior mes cells marked at E12.5 do not expand normally and this results in a smaller inferior colliculus in the adult. By contrast, the anterior r1 cells marked at E12.5 are not only retained but contribute to more lateral regions of the vermis than normal. If the lineage restriction at the...
mes/r1 border that restricts mes and r1 cells from mixing (Zervas et al., 2004) is disrupted in En1/2 mutants, then it is possible that the population of marked mes cells in En1CreERT1/+;En2–/–;R26R/+ embryos move into r1 and expand the marked population in the Cb. Another possibility is that the precursors of the lateral Cb are selectively lost in the mutants. However, this is not in accordance with our observation that the hemispheres of En1–/+;En2–/– adults are less compromised than the vermis. Although the ultimate overall loss of cells in the mes and r1 of En1–/+;En2–/– mutants could be accounted for by cell death, similar to the situation in En1 mutants (Chi et al., 2003), our fate mapping study shows that it is not as simple as the cells being lost equally on either side of the isthmus.

**En1 is required after E9 only for development of the inferior colliculus**

Consistent with the En1 expression domain being encompassed by the En2 domain after E9, we found that En1CreERT1/+;En2–/–;R26R/+ conditional mutants are viable and have a normal Cb and superior colliculus. However, despite strong expression of En2 in the posterior mes after E9 when En1 is deleted in these mutants, the inferior colliculus does not develop normally in En1fl/Cre mutants. These results indicate that the En1 protein has a different function from En2, or that En1 and En2 are expressed differently in the tectum after E9. We demonstrated that the latter is the case, because the tectum develops normally when En2 is produced from the En1 locus in the absence of endogenous En2 (En1En2ki/En2ki;En2–/– mice). Based on our expression analysis, the crucial difference must be that En1 is transiently expressed around E9 in a broader domain than En2, or that En1 is later produced at a higher level than En2. Regardless, our studies have uncovered a differential requirement for the two En genes in the superior and inferior colliculi (see Table 1 and Fig. 8A).

**En1 and En2 are differentially required in subregions of the Cb**

Our analysis of En1/2 double-mutant combinations (null, knock-in and conditional) uncovered additional differential requirements for En1 and En2 in specific regions of the Cb (see Table 1 and Fig. 8B). En1/2 functions are normally uncoupled in the hemispheres as only En2 is required to divide the posterior region into two folds (crusII and paramedian). However, the partial rescue of the hemisphere
phenotype in rare $\text{En1}^{+/+};\text{En2}^{+/+}$ and $\text{En1}^{\text{floxed}/\text{Cre}};\text{En2}^{+/+}$ mutants indicates that En1 can support hemisphere development when expressed more laterally than normal. A comparison of the phenotypes of these mice with $\text{En1}^{\text{floxed}/\text{Cre}};\text{En2}^{+/+}$ mutants (which have normal posterior foliation) indicated that En2 plays a greater role than En1 in formation of folium VIII. We demonstrated that this difference is not owing to a difference in gene expression, but instead to a difference in protein activity because the vermis foliation defect seen in $\text{En1}^{+/+};\text{En2}^{-/-}$ mutants is rescued in $\text{En1}^{\text{floxed}/\text{Cre}};\text{En2}^{+/+};\text{En2}^{+/+}$ mice. Furthermore, $\text{En1}^{\text{floxed}/\text{Cre}};\text{En2}^{-/-}$ mice have a milder phenotype than $\text{En1}^{+/+};\text{En2}^{-/-}$ mutants. Thus, En2 appears to be more effective in promoting development of the vermis (folia I-V and VIII) than En1.

We further discovered that the two En genes act concomitantly to divide the anterior Cb into five folia. $\text{En1}^{+/+};\text{En2}^{+/+}$ double heterozygotes and the majority of $\text{En1}^{\text{floxed}/\text{Cre}};\text{En2}^{+/+}$ mutants have a fusion of the anterior three folia (I-III) and the anterior defect is greatly exaggerated in $\text{En1}^{+/+};\text{En2}^{-/-}$ mutants (fusion of folia I-V), despite En2 mutants having normal anterior foliation. Since some $\text{En1}^{\text{floxed}/\text{Cre}}$ mice have normal anterior folia, this indicates a crucial requirement for expression of $\text{En1}$ only at ~E9, when $\text{En1}$ expression is fading out in the mutants and $\text{En2}$ is initiating. To our knowledge, this is the first evidence that the pattern of Cb folia can be influenced by genetic events that occur at such an early embryonic stage.

It is revealing to compare the early $\text{En1}/2$ expression patterns and the broad regions of the mes/r1 that differentially require the two genes. Based on our recent fate map of the mes/r1 using GIFM (Sgaier et al., 2005; Zervas et al., 2004), the anterior and posterior mes give rise to the superior and inferior colliculi, respectively (Fig. 8A). Consistent with strong and sustained expression of the En genes in the primordium of the inferior colliculus, this region is most sensitive to the dose of En genes, and in particular to that of $\text{En1}$. However, by using the sensitive assay of $\text{En1}^{+/+}$ knock-in alleles combined with removal of the endogenous En2 gene, we found that En2 is at least as potent as En1 at promoting inferior colliculus development. Given the transient expression of the En genes in the superior colliculus, it is perhaps surprising that this region is dependent at all on the combination of the two genes. This indicates a requirement for a short burst of En function (En1 or En2) before E9.5. The remaining inferior colliculus tissue in En mutants is likely to correlate with tectum cells that are normally in the low end of the En gradient, suggesting that they are least sensitive to loss of En alleles. There also is a general correlation between the domains of En gene expression and the requirement for each gene in the Cb (vermis versus hemispheres). After E9.5, $\text{En1}$ is only maintained in anterior r1 and the medial Cb primordium (the anlage of the vermis), consistent with no function in the hemispheres. The limit of the En2 expression domain extends more posterior early in r1 and laterally later in the CbP, correlating with a role in the hemispheres. It is not clear, however, why the En genes do not play a major role in the anterior hemispheres or in folia VI/VII and IX/X in the vermis.

**An ‘En code’ divides the tectum and Cb into subregions**

Taken together, our analysis of a series of En mutants provides evidence that functional domains of the Cb are genetically encoded by the engraved genes, as specific regions of the tectum and Cb have differential sensitivities to reducing En gene dosage. The phenotypes of multiple mutants point to a genetic division of the tectum into two regions and of the Cb into six. We propose that this represents an ‘En code’ that is used to partition the mes/r1 region into domains that in the adult regulate related neural functions (Fig. 8B). The two functional divisions of the tectum, the inferior and superior colliculi, are delineated based on a temporal requirement for $\text{En1}$ and sensitivity to the overall dose of En protein. Within the vermis of the Cb, the anterior five folia (I-V) and folium VIII are particularly sensitive to a reduction of En genes, and preferentially to En2, thus dividing the vermis into four broad regions (folia I-V, VI/VII, VIII, IX/X). Strikingly, this division of the Cb is very similar to the transverse zones recently proposed based on four different domains of parasagittal gene expression (Armstrong et al., 2005; Ozol et al., 1999). The fact that two independent genetic measures of regionalization of the vermis (mutant phenotypes and gene expression) point to the same subdivisions of the vermis strongly argues that patterning of the folia is fundamental to organization of Cb function. Consistent with this, each transverse zone receives afferent inputs from distinct regions of the spinal cord and/or particular hindbrain nuclei. We predict that, likewise, the division of the hemispheres into regions based on a need for En2 only in two folia (crusII and paramedian) (Fig. 8B) represents genetic partitioning into related functional systems.
Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/12/2325/DC1

References