Engrailed Homeobox Genes Regulate Establishment of the Cerebellar Afferent Circuit Map

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The spatial organization of the cerebellar afferent map has remarkable correspondence to two aspects of intrinsic patterning within the cerebellum embodied by a series of lobules and Purkinje cell (PC)-striped gene expression. Using male and female mice, we tested whether the *Engrailed* (*En*) homeobox genes are a common genetic substrate regulating all three systems, since they are expressed in spatially restricted domains within the cerebellum and are critical for patterning PC gene expression and foliation. Indeed, we discovered that *En1/2* are necessary for the precise targeting of mossy fibers to distinct lobules, as well as their subsequent resolution into discrete parasagittal bands. Moreover, each *En* gene coordinately regulates afferent targeting and the striped pattern of PC protein expression (e.g., ZebrinII/AldolaseC) independent of regulating foliation. We further found that *En1/2*, rather than the presence of a full complement of lobules, are critical for generating PC protein stripes and mossy fiber bands, and that PC striped gene expression is determined before afferent banding. Thus, the *En* transcription factors not only regulate cerebellum circuit topography, but they also link afferent and efferent neurons precisely enough that alterations in PC protein expression can be used as a read out for underlying defects in circuitry. In summary, our data suggest that *En1/2* are master regulators of three-dimensional organization of the cerebellum and coordinately regulate morphology, patterned gene expression, and afferent topography.

Introduction

The adult cerebellum (Cb) is organized into functional circuits that have spatially ordered afferent projections. Since the finding that expression of many proteins in Purkinje cells (PCs), for example ZebrinII/AldolaseC, is organized into striking parasagittal stripes that are mirrored by afferent activity, a critical question to answer has been whether there is a direct relationship between transcription factors within the Cb and the organization of the underlying neural circuitry. The Cb has two major afferent pathways, mossy fibers and climbing fibers, which carry sensory–motor information to the Cb from diverse regions of the brain and spinal cord (Ito, 2006). Importantly, functionally distinct afferent systems project to specific subsets of lobules along the anterior–posterior (AP) axis (numbered I–X) (Larsell, 1952), and resolve into parasagittal domains in the medial–lateral (ML) axis that mirror the PC parasagittal stripes of protein expression (Apps and Hawkes, 2009). Moreover, imaging and electrophysiological studies have demonstrated that functional activity is organized into parasagittal domains in particular lobules that

correlate spatially with PC gene expression (Chockkan and Hawkes, 1994; Ebner et al., 2005; Wadiche and Jahr, 2005; Schonewille et al., 2006). Although *in vitro* assays have implicated some extracellular proteins in guiding Cb afferents, no master regulator of three-dimensional organization of afferent topography has been identified.

One possibility is that a common pathway regulates the patterning of Cb circuitry, morphology, and PC gene expression, since the afferent topography correlates with the intrinsic subdivisions set down by the lobules and PC parasagittal protein stripes. We hypothesized that a candidate master regulator of spatial cues in the Cb is the *Engrailed* (*En*) homeobox transcription factor family, since it is critical for patterning lobules and PC protein stripes (Kuemerle et al., 1997; Sgaier et al., 2007; Sillitoe et al., 2008; Cheng et al., 2010). Furthermore, *En1/2* are among several genes transiently expressed in distinct ML domains beginning around embryonic day (E) 15.5 in the mouse (Millen et al., 1995), and the PCs expressing such genes have a defined fate with respect to adult PC stripes (Sillitoe et al., 2009). In addition, the patterning of lobules and parasagittal PC gene expression is altered independently in some *En1/2* mutants, indicating that the *En* genes regulate the patterning of morphology and PC striped gene expression by distinct processes (Sillitoe et al., 2008). A key question that has not been answered is whether *En1/2* also play a role in organizing Cb circuitry. Importantly, alterations in foliation and/or PC gene expression might then be a useful indicator of underlying defects in circuitry.

We show here using an allelic series of mutant mice that *En1/2* act within the Cb to regulate the correct targeting of three mossy

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fiber systems to particular subsets of lobules, as well as their post-natal resolution into ML bands. Importantly, each *En* gene has a dominant function in targeting afferents of particular circuits that correlates with the role of each gene in patterning PC gene expression, and not foliation. Moreover, in *Gli2* conditional mutants, which have a simplified foliation pattern, both PC protein stripes and afferent topography are properly patterned. Additionally, changes in the patterns of PC protein stripes were found to precede disruptions in afferent topography. Our *in vivo* genetic data thus demonstrate a key role for *En1/2* in establishing topographic connections within the Cb circuit map, and that PC protein stripes can be used as a readout of afferent topography.

Materials and Methods

Mice. All animal studies were carried out under an approved International Animal Care and Use Committee animal protocol according to the institutional guidelines at New York University School of Medicine and Memorial-Sloan Kettering Cancer Center. Two *En2* null alleles [*hd* (Joyner et al., 1991) and *ntd* (Millen et al., 1994)], three *En1* null alleles [*hd* (Wurst et al., 1994), *cre* (Kimmel et al., 2000), *creER^{TR}* (Sgaier et al., 2005)], and an *En1* conditional allele [*flox* (Sgaier et al., 2007)] were used and genotyped as described. *En1^{cre/+};Gli2^{flox/zf/d}* conditional mutants (*En1^{cre/+};Gli2^{flox/-}*) were maintained and genotyped as previously described (Corrales et al., 2006). The mutants were kept on an outbred background except for *En2^{hd}* mutants that have been bred to C57BL/6.

Histology and immunohistochemistry. Immunohistochemistry for ML stripe markers was performed as described previously (Sillitoe et al., 2003, 2008) with the exception of anti-Sst28 (Millipore Bioscience Research Reagents), which was used at 1:1000.

Anterograde tracing. Anterograde tracing of spinocerebellar mossy fibers using wheat germ agglutinin (WGA)-HRP (Sigma) was performed as previously described (Vogel et al., 1996). Note the minor variability in labeling between animals. The variability typically depends on the size of the injection, the amount of tissue damage caused at the injection locus, the capacity of the tissue to recover after surgery, and the efficiency of tracer transport. Despite these variables, which we try to control with standardized injection sizes, carefully controlled delivery of the tracer with finely pulled electrophysiological pipettes, and sterile surgical technique, there was still a modest level of variability in the number of tetramethyl benzidine (TMB; Sigma)-stained axons observed between animals. Substantial variation in labeling arose only after poor injections, which resulted in an almost complete lack of staining. Retrospective analysis of the injection spot confirmed that in most of these cases the injection caused too much tissue damage. Such mice were not included in the final analyses. Injections that showed extensive labeling, but with limited variation in banding patterns observed between animals, suggest that the different patterns of termination arose due to phenotypic differences in *En1/2* mutants. Importantly, we recently reported that deletion of *En1/2* results in PC stripe patterning defects, which can vary slightly between animals of the same genotype (Sillitoe et al., 2008). Thus, although the basic phenotype is seen in every animal, the overall density of stained afferents can vary. Occasional background staining due to leakage of WGA-HRP into the CSF was observed in the molecular layer after TMB histochemistry (see Fig. 3*d*, white arrows).

Microscopy and data analysis. Photomicrographs were captured using a Retiga SRV camera (QImaging) mounted on a Leica DM6000 microscope. Images were acquired and analyzed using Velocity software (version 4.1.0; PerkinElmer) and thereafter imported into Adobe Photoshop CS2 and Adobe Illustrator CS2.

Results

In the adult vermis, the expression of many proteins, including ZebrinII/AldolaseC (Brochu et al., 1990; Ahn et al., 1994) and the small heat shock protein Hsp25 (Armstrong et al., 2000), is restricted to distinct patterns of PC parasagittal stripes in four transverse zones along the AP axis (Ozol et al., 1999): the anterior zone (AZ; lobules I–V), the central zone (CZ; lobules VI and VII),

Figure 1. Molecular coding and foliation form a two-coordinate system in the Cb. *a, b*, Gene expression domains in the ML axis divide the 10 lobules into four AP transverse zones. Lobules I–V (red) constitute the AZ, lobules VI–VII (blue) the CZ, lobules VIII–anterior IX (green) the PZ, and lobules posterior IX–X (black) the NZ. *c*, The ML patterns of ZebrinII and Hsp25 uniquely identify each transverse zone. *d*, Examples of ZebrinII (AZ) and Hsp25 (CZ) ML stripe patterns in the adult mouse Cb. *e*, Double labeling for ZebrinII (brown, diaminobenzidine) and WGA-HRP (black, nickel-enhanced TMB signal) in lobule VIII of an *En1^{flox/cre}* mouse showing the common ground plan of PC stripes and spinocerebellar mossy fiber bands in the Cb. Note that ZebrinII expression is restricted to PC somata in the Purkinje cell layer (pcl) and dendrites in the molecular layer (ml), whereas mossy fibers terminate exclusively within the granule cell layer (gcl). In all figures, lobules are indicated by Roman numerals and the stripe nomenclature was previously described (Brochu et al., 1990; Armstrong et al., 2000). A, Anterior; P, posterior; M, medial; L, lateral. Scale bar: *e*, 50 μ m.

the posterior zone [PZ; lobules VIII, anterior (a) IX], and the nodular zone [NZ; lobules posterior (p) IX, X] (Fig. 1*b–d*). Importantly, although PC stripe gene expression is most widely studied, gene expression in Cb cortical interneurons and axon

Figure 2. ML topography of spinocerebellar mossy fibers is severely disrupted in *En1/2* mutants. **a, c, e**, ZebrinII stripes are severely altered in the PZ of *En* mutant mice. **b, d, f**, Spinocerebellar mossy fiber topography is disrupted in the PZ of *En1/2* mice and is correlated with changes in molecular coding. **d**, In *En1^{flox/cre}* mutant mice lobule aVIII contains several small clusters (arrows), and the dense staining in pVIII disrupts the clear banding pattern. **f**, In *En1^{+/-};En2^{-/-}* mutants, one large mossy fiber terminal field was located at the midline with two large fields located laterally. **g–l**, Topographical changes in the ML mossy fiber pattern in the AZ of *En1^{flox/cre}* and *En1^{+/-};En2^{-/-}* mutant mice. In *En1^{+/-};En2^{-/-}* mutant mice, bands with ectopic terminals are denoted with red asterisks (**l**). The brackets in **j** and **j** indicate the limits of S2, and the arrow points to the thinner ZebrinII and matching S2 band in *En1^{flox/cre}* mice. The arrows in **k** point to the fragmented P1 + to P3 + ZebrinII stripes in *En1^{+/-};En2^{-/-}* mice. The dotted lines on the sagittal schematics indicate the level of where the coronal tissue sections were taken from. Lobules are indicated by Roman numerals. Scale bar: **a** (for **a–h**), 500 μ m.

tracts has been shown to share a common topography with PCs (Apps and Hawkes, 2009). Adult parasagittal gene expression as a whole thus provides a molecular code for the spatial organization of the Cb (Sillitoe and Joyner, 2007). In *En1/2* double mutants, the adult PC molecular code is disrupted in all four zones; whereas, the lobules are preferentially lost in the AZ and PZ (Sgaier et al., 2007; Sillitoe et al., 2008; Cheng et al., 2010). In addition, whereas *En2* is dominant over *En1* in patterning the lobules, *En1* plays a dominant role in patterning the molecular code in the AZ and PZ (Sgaier et al., 2007; Sillitoe et al., 2008). Thus, the *En* genes can regulate patterning of lobule morphology and molecular coding by separate processes. In the following experiments, we use genetic approaches to determine whether *En1/2* also play a role in organizing Cb circuitry.

En1/2 regulate ML afferent topography in the Cb

As a first approach to test whether afferent topography is dependent on molecular coding and/or foliation processes regulated by *En1/2*, we analyzed mossy fiber topography in *En1^{flox/cre}* condi-

tional mutants, which lack *En1* function after E9, as they have normal morphology but disrupted molecular coding in the AZ (Fig. 2*a,c,e*) and PZ (Fig. 2*g,i,k*) (Sgaier et al., 2007; Sillitoe et al., 2008). We began by examining the spinocerebellar mossy fiber system, since it projects only to the AZ and PZ in the vermis. Mossy fibers that originate from the spinal cord terminate as large synaptic glomeruli in specific ML bands that align with ZebrinII PC stripes in the AZ and PZ (Vogel et al., 1996; Apps and Hawkes, 2009). Axon collaterals project between the two zones and respect the same fundamental band pattern as the primary mossy fibers (Heckroth and Eisenman, 1988). Strikingly, the banded organization of the spinocerebellar terminal field was disrupted in both the AZ and PZ of *En1/2* mutants, and the degree of alterations correlated with changes in the molecular code. In the PZ vermis of wild-type (WT) mice, two pairs of afferent bands (S1 and S2) (Fig. 2*b*) are located on either side of the midline in the posterior part of lobule VIII (pVIII), and there is only one pair (S1) on the anterior face of IX (aIX). In contrast, in lobule pVIII/aIX of *En1^{flox/cre}* mutants the topography of mossy fibers was drastically altered, with a midline band and many ectopic terminals located throughout the lobule. Strikingly, the lack of pattern correlated with the diffuse stripes of ZebrinII expression in pVIII (Sillitoe et al., 2008) (Fig. 2*c*). Curiously, in aVIII of *En1^{flox/cre}* mutants three narrow but reproducible ($n = 8/8$) mossy fiber bands occupied the midline (Fig. 2*d*, arrowheads), compared with only a few scattered spinocerebellar terminals in controls (Fig. 2*b*). Although the pattern was reproducible between mutants ($n = 8/8$), the intensity of labeling differed (compare Fig. 2*d*, supplemental Fig. 2*h*,

available at www.jneurosci.org as supplemental material), likely due to differences in the efficiency of WGA-HRP transport. Interestingly, this gain of mossy fiber patterning in aVIII correlates with ZebrinII expression having a more complex pattern in aVIII of *En1^{flox/cre}* mutants compared with WT mice (Sillitoe et al., 2008). In addition, ectopic terminals densely populated the hemisphere extension of lobule VIII (copula pyramidis), where fewer spinocerebellar mossy fibers normally project (Fig. 2*b*, black asterisk; Fig. 2*d*, yellow asterisk; supplemental Fig. 2*h*, available at www.jneurosci.org as supplemental material, arrows).

In the AZ of WT mice, spinocerebellar mossy fibers terminate in one midline (S1) and two adjacent symmetrical pairs of bands (S2 and S3) (Fig. 2*h*). In *En1^{flox/cre}* mutants ($n = 8$), the S1 band was weakly stained and the S2 band was often wider than in WT mice (Fig. 2*j*). The presence of a wider S2 band always correlated with the presence of an ectopic ZebrinII stripe (Fig. 2*i,j*) (Sillitoe et al., 2008). Thus, in *En1^{flox/cre}* mutants, even though the morphology and size of the Cb are largely normal, the ML topography of mossy fiber bands is disrupted, and the degree of disruption

correlates with the degree of molecular code defects. In addition, in the PZ of the vermis the limits of the projection domain ectopically expanded anteriorly and laterally.

En2^{−/−} mice have only mild defects in spinocerebellar mossy fiber organization (data not shown) (Vogel et al., 1996) and subtle changes in ZebrinII expression (Sillitoe et al., 2008). We tested whether *En1/2* together regulate ML mossy fiber topography by analyzing *En1*^{+/−};*En2*^{−/−} mutants. Indeed, mossy fiber topography was markedly altered in what remained of the lobules in the AZ of *En1*^{+/−};*En2*^{−/−} mice (*n* = 6). The midline pair of bands (S1) in *En1*^{+/−};*En2*^{−/−} mutants was more heavily deposited with WGA-HRP than normal (Fig. 2*l*), and the interbands contained many terminals (Fig. 2*l*, red asterisks; supplemental Fig. 1*i*, available at www.jneurosci.org as supplemental material). In addition, whereas in lobules I–III of the AZ in WT mice S1 was less dense than S2 (Fig. 2*h*), the reverse was found in the AZ of *En1*^{+/−};*En2*^{−/−} mutant mice such that S1 was substantially denser and wider than S2 (Fig. 2*l*; supplemental Fig. 1*i*, available at www.jneurosci.org as supplemental material). In the PZ of *En1*^{+/−};*En2*^{−/−} mutant mice where lobule VIII is greatly reduced, one wide medial band and two lateral bands replaced the normal two lateral pairs of bands (Fig. 2*f*). As in the AZ, the intensity of HRP histochemical staining, and thus likely the density of terminals, was increased in *En1*^{+/−};*En2*^{−/−} mutant mice compared with WT mice (Fig. 2*f*, lobule VIII/aIX). In summary, the disrupted mossy fiber topography reflected the altered ZebrinII molecular coding with fragmentation in the AZ (Fig. 2*k,l*) and fused midline mossy fiber bands in the PZ (Fig. 2*e,f*).

To further probe how *En1* and *En2* together regulate afferent topography, we analyzed *En1/2* single and double heterozygotes. We found that, similar to molecular coding, afferent topography was more sensitive than foliation to the dosage of *En1/2* (Fig. 3; supplemental Fig. 1, available at www.jneurosci.org as supplemental material), as *En1*^{+/−} mice had mild, but consistent defects in the AZ and PZ (Fig. 3*e*, arrow; 3*f*, red brackets and asterisk), and *En2*^{+/−} mice in the PZ. Moreover, the severity of afferent targeting defects increased in the allelic series in the same order as ML molecular coding defects: *En2*^{+/−} < *En1*^{+/−} < *En2*^{−/−} < *En1*^{+/−};*En2*^{+/−} < *En1*^{flox/cre} < *En1*^{+/−};*En2*^{−/−} mutant mice (Fig. 3; supplemental Fig. 1, available at www.jneurosci.org as supplemental material).

A reduction in the number of lobules does not necessarily disrupt mossy fiber topography

One possible contribution to the disruption of afferent patterning in *En1*^{+/−};*En2*^{−/−} mutants is the smaller Cb and thus reduced target field. To test whether a reduction in lobules necessarily leads to a disruption of mossy fiber topography, we examined afferent topography in *En1*^{cre/+};*Gli2*^{flox/−} conditional knock-out

Figure 3. The adult pattern of mossy fiber bands is sensitive to single-copy deletions of *En1* and *En2*, but not to removal of *Gli2*. *a–d*, The pattern of spinocerebellar mossy fiber termination in the AZ and PZ of *En2*^{+/−} mice is similar to WT mice (compare with Fig. 2*b,h*). The white arrows in *d* point to nonspecific labeling due to leakage of WGA-HRP into the CSF during surgery (also see Materials and Methods). *e, f*, In contrast, in *En1*^{+/−} mice, S1 in the AZ (*e*, white arrow) was depleted of terminals, and S1 and S2 were fused in the PZ (inverted red brackets in *pVIII*). *g, h*, *En1*^{cre/+};*Gli2*^{flox/−} mutants had an essentially normal pattern, and the depletion of S1 in the AZ (black arrow), ectopic terminals in the copula pyramidis (cop) as shown in the inset of *h*, and fusion of S1 and S2 in the PZ were attributed to the lack of one copy of *En1* in these mice. The image in the inset of *h* was taken from a near adjacent tissue section. The dotted lines on the sagittal schematics indicate the level from which the coronal tissue sections were taken. Scale bar: (in *h*), *a–h*, 1 mm; inset, 2 mm.

mutants, which have an approximately one-third reduction in the size of the Cb. *Gli2*, a transcription factor post-translationally regulated by Sonic hedgehog (Shh) signaling, is expressed in the Cb beginning at ~E15.5 in all cells but the PCs that express Shh (Corrales et al., 2004). In *En1*^{cre/+};*Gli2*^{flox/−} mice granule cell proliferation is reduced, and the foliation pattern resembles an immature postnatal day (PD) 2 WT Cb with only five major folds (Corrales et al., 2006). Thus, patterning of the lobules can be considered normal, but Cb development is halted prematurely.

We first analyzed molecular coding in *En1*^{cre/+};*Gli2*^{flox/−} mice (*n* = 10) to determine whether it was also normal. Indeed, the expression patterns of ZebrinII (Fig. 4*a,c,e,g*) and Hsp25 (Fig. 4*b,d,f,h*) were found to be normal in the AZ/PZ and CZ/NZ, respectively. Thus, dramatic defects in lobule morphogenesis do not necessarily impact the formation of a normal molecular code.

Correlating with the normal PC stripes, five clear ML spinocerebellar mossy fiber bands were detected in the AZ of *En1*^{cre/+};*Gli2*^{flox/−} mice (*n* = 10) with no increase in terminals between bands (Fig. 3*a,g*). Like *En1*^{+/−} controls, the S1 band had a reduced number of mossy fiber terminals (Fig. 3*e*, white arrow; Fig. 3*g*, black arrow). In lobule pVIII of the PZ (Fig. 3*h*), the pattern also resembled *En1*^{+/−} controls (*n* = 4) (Fig. 3*f*) with one wide midline mossy fiber band replacing the normal two bands (Fig. 3*b*). *En1*^{cre/+};*Gli2*^{flox/−} mutants also had a similar pattern to the controls in aIX (Fig. 3*f,h*). Finally, *En1*^{cre/+};*Gli2*^{flox/−} mutants like *En1*^{+/−} controls had ectopic terminals in the copula pyramidis (Fig. 3*f*, red arrow; Fig. 3*h*, inset, white arrow). Thus, decreasing

Figure 4. Molecular coding is normal in *En1^{cre/+};Gli2^{flox/-}* mutant mice. **a–d**, ZebrinII molecular coding as seen on coronal cut tissue sections in the AZ (**a**) and PZ (**c**), and Hsp25 molecular coding in the CZ (**b**) and NZ (**d**). **e–h**, ZebrinII (**e, g**) and Hsp25 (**f, h**) molecular coding is normal in all four transverse zones in *En1^{cre/+};Gli2^{flox/-}* mutant mice. The sagittal schematics on the left (WT) indicate the lobules that show parasagittal stripes of ZebrinII (red) and Hsp25 (blue), and correspond to the tissue sections shown in **a–d**. The sagittal schematics on the right (*En1^{cre/+};Gli2^{flox/-}*) indicate the lobules that show parasagittal stripes of ZebrinII (red) and Hsp25 (blue), and correspond to the tissue sections shown in **e–h**. The dotted lines on the sagittal schematics indicate the level from which the coronal tissue sections were taken. Scale bar: **a** (for **a–h**), 500 μ m.

Shh/Gli signaling in the Cb and dramatically reducing the size of the Cb does not alter the basic ML topography of spinocerebellar afferents or molecular coding.

En1/2 are required to restrict afferent termination into distinct AP domains

The presence of ectopic terminals in aVIII in *En1^{flox/cre}* mutants raised the question of whether *En1/2* regulate targeting of mossy fibers in the AP dimension, in addition to their clear role in ML targeting. Consistent with this idea, in the posterior Cb of *En1^{+/-};En2^{-/-}* mutants spinocerebellar afferents projected ectopically not only into lobule aVIII, but also into a more posterior region of aIX than normal (Fig. 5*c–e*, brackets). A similar, but milder, posterior expansion of the PZ terminal domain also was observed in *En1^{flox/cre}* mice (Fig. 2*b,d*; supplemental Fig. 1*h*, available at www.jneurosci.org as supplemental material). Moreover, in both mutants the expansion of terminal fields in the AP axis correlated with an expansion of molecular patterning (Fig. 5*i,k*). Furthermore, in the anterior vermis of *En1^{+/-};En2^{-/-}* ($n = 4$) (Fig. 5*b,e,h*), but not *En1^{flox/cre}* (data not shown) mutant mice the spinocerebellar mossy fiber termination field expanded posteriorly into the anterior face of lobule VI (Fig. 5*f–h*, bracket). In addition, in lobule VIa of *En1^{+/-};En2^{-/-}* mutants, ZebrinII stripes are expanded posteriorly into this region (Fig. 5*i–k*) (Sillitoe et al., 2008). Since foliation is normal in the posterior Cb of *En1^{flox/cre}* mutants (Fig. 2; supplemental Fig. 1, available at www.jneurosci.org as supplemental material) (Sgaier et al., 2007), the expansion of the PZ termination field must be attributed to the loss of *En1* function in afferent patterning and not changes in the number of target neurons. Since *En1^{+/-};En2^{-/-}* mutants have fewer lobules, one possibility was that afferents misproject

outside their termination field in these mutants due to the overall reduction in the size of their target field, rather than guidance being altered as reflected by abnormal molecular coding. If this were the case, then mossy fiber patterning in the AP axis should be more severely affected in *En1^{cre/+};Gli2^{flox/-}* mutants than in *En1^{+/-};En2^{-/-}* mice. Contrary to this prediction, the AP patterning of spinocerebellar afferents was more severely affected in *En1^{+/-};En2^{-/-}* mice (Fig. 5). These results argue that the size of the Cb in *En1^{+/-};En2^{-/-}* mutants does not account for the extensive mistargeting of afferents to adjacent lobules, but instead *En1/2* regulate mossy fiber topography in the AP orientation, in addition to the ML orientation.

En1/2 are required for orchestrating the development of multiple sensory maps

En2^{-/-} mice have subtle defects in spinocerebellar mossy fiber topography (Vogel et al., 1996). However, given that both molecular coding and afferent topography defects were detected in lobule VIII of the PZ, which has abnormal foliation, it was not clear whether the circuitry defects were secondary to foliation. To determine whether *En1/2* play a broad role in regulating mossy fiber topography, we analyzed the CZ and NZ topography of a subset of mossy fibers that project from the pontine and vestibular nuclei, and express Somatostatin28 (Sst28) (Yacubova and Komuro, 2002). In WT mice, Sst28 marked distinct subsets of mossy fiber terminals in the internal granular layer (IGL) of lobules pIX and X (the vestibulocerebellar domain) (Figs. 6*a, 7b*) (Armstrong et al., 2009) and lobules VIb and VII (the pontocerebellar domain) (Fig. 7*a*). Although a clear ML Sst28 pattern was not observed in the CZ, in pX of the NZ four clear ML bands of Sst28 were present: one pair on either side of

the midline (Fig. 6*a*, rectangle and arrow) and an additional pair at the lateral edges (data not shown) (Armstrong et al., 2009). Double immunostaining in WT mice for choline acetyltransferase, which in the vermis marks vestibular mossy fibers that project mainly to the NZ (Barmack et al., 1992; Sillitoe et al., 2003), showed that ~50% of the Sst28 immunoreactive terminals in the NZ likely belong to mossy fibers of the vestibulocerebellar tract (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).

In mutants lacking *En1* ($n = 8$), there was a substantial reduction in the number of immunostained terminals in each NZ band (Fig. 6*b*, white rectangle). Conversely, in *En2*^{-/-} (Fig. 6*c*, arrow) ($n = 8$) and *En1*^{+/-};*En2*^{-/-} (data not shown; $n = 8$) mutants, ectopic terminals were located at the midline, producing a uniform domain across the midline. In sagittal sections, it was apparent that the NZ domain was shifted posteriorly in *En2*^{-/-} ($n = 4$) and *En1*^{+/-};*En2*^{-/-} ($n = 4$) mutants (Fig. 7*f,h*, arrows). As in the NZ, the number of terminals in the CZ of *En1*^{flox/cre} mutants ($n = 4$) was severely reduced (Fig. 7*a–d*), but the number of terminals in *En1*^{+/-};*En2*^{-/-} mutants was also reduced (Fig. 7*g*). In *En2*^{-/-} mutants, the number of terminals was not greatly reduced in the CZ domain (Fig. 7*e*), but the domain appeared slightly expanded anteriorly (Fig. 7, compare *a* and *e*, positions of arrows; supplemental Fig. 3, available at www.jneurosci.org as supplemental material, arrows). We also observed scattered Sst28-immunoreactive terminals in the IGL of the anterior face in lobule VIa (Fig. 7*e*, arrowheads; supplemental Fig. 3, available at www.jneurosci.org as supplemental material, arrowheads). We observed very little variability in the staining pattern of Sst28 between animals of the same genotype, and we therefore attribute any differences in mossy fiber patterning to each phenotype. Together, these results demonstrate that *En1* and *En2* regulate different aspects of development of multiple mossy fiber systems that send sensory information to distinct AP and ML zones. Moreover, the AP and ML patterning defects in mossy fiber topography in the CZ/NZ of *En2*^{-/-} mice are accompanied by disruptions in the molecular code (Hsp25) (Sillitoe et al., 2008).

En1/2 are required for patterned axon regression postnatally after molecular coding is established

En1/2 could be required to establish the specific ML band patterns of mossy fibers, or to maintain properly patterned topography. To distinguish between these possibilities, we determined the normal process of spinocerebellar terminal field development and then analyzed the process in *En1*^{+/-};*En2*^{-/-} mice as they have the most disrupted patterns in the AZ and PZ. Spinocerebellar mossy fibers enter the mouse Cb at approximately E13/14 (Grishkat and Eisenman, 1995). Previous studies suggest that the adult band pattern is established by approximately PD7 in rat

Figure 5. Spinocerebellar mossy fibers ectopically project into neighboring domains in *En1*^{+/-};*En2*^{-/-} mutant mice. *a, b*, Spinocerebellar mossy fibers terminate in the AZ (lobules I–V) and PZ (lobules VIII/IX) in WT (*a*) and *En1*^{+/-};*En2*^{-/-} (*b*) mice as seen on sagittal cut tissue sections. The regions indicated by the brackets are shown at higher magnification in *a'* and *b'*. *c, d*, Spinocerebellar mossy fibers terminate predominantly in the posterior aspect of lobule VIII and the anterior aspect of lobule IX (rostral half) in WT and *En1*^{cre/+};*Gli2*^{flox/+} mutants. *e*, In *En1*^{+/-};*En2*^{-/-} mutant mice, spinocerebellar mossy fiber terminals are densely distributed in the anterior aspect of lobule VIII and throughout lobule IX. The red arrows indicate the base of the fissure between lobules VIII and IX. *f–h*, Coronal cut tissue sections showing the absence of spinocerebellar mossy fibers in lobule VI of WT (*f*) and *En1*^{cre/+};*Gli2*^{flox/+} mutants (*g*) and the presence of spinocerebellar mossy fibers at the midline of lobule VI in *En1*^{+/-};*En2*^{-/-} mutant mice (*h*). *i–k*, ZebrinII is expressed by most PCs of the CZ in WT (*i*) and *En1*^{cre/+};*Gli2*^{flox/+} mutants (*j*) (arrowheads) but is expressed in stripes (arrowhead) in the CZ of *En1*^{+/-};*En2*^{-/-} mutant mice (*k*). *a*, Anterior; *p*, posterior. Scale bars: *b* (for *a, b*), 1 mm, (for *a', b'*), 250 μ m; *k* (for *c–j*), 1 mm.

(Arsênio Nunes and Sotelo, 1985). At birth, spinocerebellar mossy fibers in WT mice were found to terminate throughout the ML extent of the AZ and PZ (data not shown). By PD3, the fibers still occupied the entire ML extent of the vermis (Fig. 8*a,b*). Interestingly, at both PD0 and PD3 mossy fibers were found in more lateral regions than in the adult, including the lateral aspects of the copula pyramidis (Fig. 8*b*). Subtle indications of heavy versus weak spinocerebellar termination domains were seen in the vermis at PD3 (Fig. 8*a*, asterisks). By PD5, a clear adult banded pattern was seen in the vermis with few terminals persisting in the hemispheres (Fig. 8*c,d*). Consistent with our analysis of mossy fiber topography in the adult *En1*^{cre/+};*Gli2*^{flox/+} mutants, both the AP and ML patterns of spinocerebellar mossy fibers observed at PD5 were like *En1*^{cre/+} (data not shown) and WT controls (Fig. 8*c–f*). In contrast, in *En1*^{+/-};*En2*^{-/-} mutants the pattern was severely disrupted at PD5 (Fig. 8*g,h*), and ectopic terminals were found in bands that are normally devoid of spinocerebellar terminals (Fig. 8*c,e,g*, black arrows). In two of six *En1*^{+/-};*En2*^{-/-} mutants, no ML bands were apparent at PD5 (data not shown). In the other animals, which had milder foliation defects, a pattern similar to the abnormal adult ML band topography was obvious, including projections throughout the copula

Figure 6. The ML topography of mossy fibers is disrupted in the NZ of *Engrailed* mutant mice. **a**, Sst28 is expressed in distinct mossy fiber bands in the NZ. Two clusters (numbered as 1) about the negative midline (arrow). Note that PCs were weakly immunoreactive for Sst28 [see Purkinje cell layer (pcl)]. **b**, **c**, In *En1^{flox/cre}* mice (**b**), there are only few mossy fibers that express Sst28 (**a** and **b**, compare the staining in the white rectangles), while in *En2^{-/-}* mice (**c**) Sst28 immunoreactive mossy fibers were ectopically located at the midline (arrow). ml, Molecular layer; gcl, granule cell layer. Scale bar: **c** (for **a–c**), 250 μ m.

pyramidis (Fig. 8*g,h*). We also found that at PD5 mossy fibers were ectopically targeted in the AP axis of *En1^{+/-};En2^{-/-}* mutants with a substantial number of mistargeted terminals in lobules VI and posterior lobule IX (data not shown).

Given the correlation between changes in molecular coding and afferent topography in *En1/2* mutants, we tested whether the molecular code is changed before PD5 in the mutants. We analyzed phospholipase C β 4 (Plc β 4) expression since, unlike ZebrinII or spinocerebellar mossy fibers, it is expressed in a clear pattern of stripes starting at approximately E18 (Marzban et al., 2007). Of additional importance, Plc β 4 expression maintains the same configuration of stripes from late embryonic stages through to adulthood (Marzban et al., 2007). Therefore, given that in the mature Cb stripes of Plc β 4 are complementary to stripes of ZebrinII (Sarna et al., 2006), early postnatal PC protein stripe defects in *En1/2* mice would be predicted to correlate to adult protein stripe defects. At PD2 in the AZ, two wide pairs of Plc β 4

stripes flank the midline (Marzban et al., 2007) (Fig. 8*i*). In contrast, at PD2 in *En1^{+/-};En2^{-/-}* mutants the Plc β 4-negative stripes were narrow and fragmented (Fig. 8*j*, arrows), and complementary to the ZebrinII pattern observed in *En1^{+/-};En2^{-/-}* adult mice (Sillitoe et al., 2008). Thus, *En1/2* are required for patterning ML molecular coding before establishment of mossy fiber topography, and the initial topographical defects of the spinocerebellar tract mirror the degree of the molecular coding defects.

Discussion

We previously proposed that the Cb contains an intrinsic coordinate system that is used to spatially organize cells, axons/dendrites, and gene expression in the AP and ML axes (Sillitoe and Joyner, 2007). We now show that the *En* transcription factors not only regulate patterning of morphology and molecular coding, but also targeting of mossy fibers within the Cb. Importantly, we observed that by gradually lowering the dose of *En1/2* changes in mossy fibers topography mirrored the severity of changes in PC protein stripe gene expression and did not correlate with disruptions of foliation. For example, despite foliation being normal in the PZ of *En1^{flox/cre}* mice, in regions where ZebrinII expression was homogeneous, mossy fibers were targeted into a uniform domain. Moreover, ectopic regions of ZebrinII striped expression in *En1^{flox/cre}* or *En1^{+/-};En2^{-/-}* mice were mirrored by AP mistargeting of spinocerebellar terminals into the CZ and NZ. Importantly, molecular coding changes were seen before development of afferent targeting defects. Thus, our data provide evidence that the molecular code is causally related to guidance cues used by incoming afferents to pattern their topography within the Cb, and can therefore be used as a readout of changes in the underlying circuit map.

Independent genetic support for the conclusion that changes in afferent topography are better reflected by changes in molecular coding than foliation come from our analysis of *En1^{cre/+};Gli2^{flox/-}* mice. Surprisingly, we found that regardless of the severe reduction in the target field of mossy fibers in *En1^{cre/+};Gli2^{flox/-}* mice, an almost normal mossy fiber topography developed in these mutants. Furthermore, the correct afferent targeting in *En1^{cre/+};Gli2^{flox/-}* mutants was accompanied by normal PC molecular coding. In accordance with our genetic studies in the Cb, ablation and transplantation of the retina and/or tectum in various species have demonstrated that compression and expansion of a target field can result in normally patterned afferent projections (Udin and Fawcett, 1988). From these data, we propose that targeting of Cb afferents is dependent on guidance cues represented by stripes of proteins in PCs in each transverse zone.

The signals required for initial organization of the PC molecular code are thought to be intrinsic to the Cb. The ML pattern of *L7/Pcp2* embryonic gene expression initiates with a normal pattern (albeit delayed) in organ cultures derived from E14 mouse Cb (Oberdick et al., 1993). In addition, ZebrinI-positive and -negative PCs develop in the absence of afferent inputs when Cb tissue from E12–E15 rat embryos (before afferents enter the Cb) is transplanted ectopically (Wassef et al., 1990). Thus, the availability of patterned gene expression within the embryonic Cb, before the arrival of afferents, provides a means by which afferents could be guided within an existing map, since the map has rudimentary features of the adult topographic circuit (Sillitoe et al., 2009).

Studies of mutant mice that have primary defects in PCs (e.g., *Staggerer*) versus granule cells (e.g., *Weaver*) suggested that PCs

could directly control the topography of developing mossy fibers (Arsénio Nunes et al., 1988). Since mossy fibers initially contact PCs in the embryonic and early postnatal Cb before synapsing with granule cells (Mason and Gregory, 1984; Manzini et al., 2006), PCs could indeed provide necessary targeting cues required to organize mossy fibers. In accordance with this idea, we found that spinocerebellar afferents do not attain their banded pattern until PD5. Furthermore, *in vitro* preparations of the embryonic chick hindbrain revealed that Cb-intrinsic molecular cues guide climbing fibers (Chédotal et al., 1997; Nishida et al., 2002; Sotelo and Chédotal, 2005). These results raised the possibility that transcription factors in the Cb regulate the expression of proteins involved in afferent targeting and formation of the Cb circuit map. Since *En1/2* are expressed in ML domains in developing PCs (data not shown) (Millen et al., 1995; Sgaier et al., 2007), *En1/2* could control the expression of PC guidance molecules critical for patterning mossy fiber topography. Consistent with this, we found that in *En1*^{+/-};*En2*^{-/-} mice the molecular code is disrupted before spinocerebellar afferents attain their mature topography. Given that *En1/2* control topographic targeting of retinotectal axons by regulating Eph/ephrin signaling (Logan et al., 1996; Brunet et al., 2005), and EphA4 might be expressed in complementary stripes to *En1/2* (Hashimoto and Mikoshiba, 2003), it is tempting to speculate that Eph/ephrins are critical targets of *En1/2* during Cb circuit formation.

En1/2 expression is dynamic during Cb development (data not shown) (Millen et al., 1995; Sgaier et al., 2007). Initially, *En1/2* mRNA and protein are expressed in the ventricular zone. During late embryonic/early postnatal Cb morphogenesis, *En1/2* are expressed in spatially restricted patterns in most cell types, including PCs and granule cells. Given that *En1/2* have multiple roles during Cb development (Sgaier et al., 2007; Sillitoe et al., 2008; Cheng et al., 2010), their dynamic expression across multiple cell types might impart specific target populations with the capacity to progressively instruct mossy fiber development. In this scenario, *En1/2* could initially regulate the expression of genes in PCs that encode for proteins that shape the overall organization of topographic circuits and later in granule cells regulate cues required for refinement of neural circuit architecture. Although the transient interaction of PCs with mossy fibers likely represents a specific mode of cell–cell communication, transient interactions during circuit formation are not unique to the Cb (Chao et al., 2009). Subplate neurons in the developing forebrain act as a transient relay between lateral geniculate neurons and the cerebral cortex (Kanold, 2009). As neurons in the cortical layers

Figure 7. The AP topography of mossy fibers is disrupted in the CZ and NZ of *Engrailed* mutant mice. **a, b**, Sst28 is predominantly expressed in lobules VIb–VII and IX–X of the mouse Cb. The asterisk in **a** indicates the fissure between lobule VIb and VII (also true for **c, e**, and **g**). The arrows indicate the anterior limits of Sst28 expression in lobule VI. **c, d**, Sst28 expression is dramatically reduced in the Cb of *En1*^{flox/cre} mice. **e, f**, In *En2*^{-/-} mice, the anterior limit of Sst28 expression in lobule VIb is expanded anteriorly, and in the NZ only a few terminals were found in lobule aX. The dashed white vertical line in **a** indicates the anterior limit of Sst28 expression in the CZ. The same line is extended into **e** for comparison with the expanded expression domain (white arrow) observed in *En2*^{-/-} mice. Note that scattered ectopic terminals were also observed in the anterior face of lobule Vla (white arrowheads). In addition, the rostral boundary of Sst28 expression in lobule aX was shifted posteriorly (**f**, arrow). **g, h**, In *En1*^{+/-};*En2*^{-/-} mice Sst28 expression is reduced in the CZ, and most immunoreactive terminals were found close to the pX (**g**, arrowheads). Compared with WT mice and *En2*^{-/-} mice, the Sst28 expression domain in the NZ was shifted posteriorly (**h**, arrows indicate the limits of the shifted domain). The schematics illustrate the location of Sst28-immunoreactive terminals in lobule VI (CZ) and lobule X (NZ). The arrows in **a** and **e** point to the anterior limits of expression in the CZ, and the asterisks indicate the intercrural fissure located between lobules VI and VII. Scale bar: **h** (for **a–h**), 200 μ m.

begin to mature, afferents from the lateral geniculate nucleus extend into the cortex and form stable synapses with their final targets in layer 4 (Kanold, 2009). Thus, in both the cerebral cortex and Cb, transient cell–cell contacts likely provide organizational cues for developing axons before the maturation of their ultimate target cells. Our studies indicate that in the Cb, *En1/2* could coordinately regulate both stages of axon targeting, since they are expressed in PCs and granule cells.

Our finding that the Cb molecular code reflects the organization of Cb circuitry has important implications for understanding human neurological diseases. Human *EN2* is one of several susceptibility loci in autism spectrum disorder (ASD) (Gharani et

Figure 8. Spino-cerebellar mossy fibers resolve into a ML banded pattern during the first postnatal week. **a, b**, WGA-HRP anterograde tracing of the spinocerebellar tract at PD3 reveals terminals in the lobules I–V (AZ) and VIII/IX (PZ) as seen on a coronal section. In the PZ mossy fiber terminals were seen throughout the entire ML extent of lobule VIII, and in the AZ a crude map of bands was observed (**a**, asterisks). **c, d**, By PD5 a clear ML banded pattern is obvious in the AZ and PZ. **e, f**, The pattern of spinocerebellar bands was normal in the Cb of PD5 *En1^{cre/+};Gli2^{lox/+}* mutants. **g, h**, The pattern of spinocerebellar bands was severely disrupted in the Cb of PD5 *En1^{+/-};En2^{+/-}* mice, with ectopic terminals located in bands that are normally devoid of spinocerebellar terminals (**c, e, g**, compare black arrows). **i, j**, In *En1^{+/-};En2^{+/-}* mice, the PC molecular code was disrupted at PD2, before spinocerebellar terminals resolve into bands, as determined by PlcB4 expression. Compared with wild-type mice (**i**), the PlcB4-positive stripes in *En1^{+/-};En2^{+/-}* mice were separated by thin negative stripes (**j**, white arrows). Scale bars: **a, b**, 250 μ m; **c–g**, 500 μ m; **i, j**, 500 μ m.

al., 2004; Benayed et al., 2005), and *En2^{+/-}* mice display neurobehavioral and neurochemical alterations typically seen in ASD (Cheh et al., 2006). Since we have uncovered that morphological defects are not necessarily associated with circuit changes (*En1^{cre/+};Gli2^{lox/+}* mutants), whereas molecular coding defects are, it should now be possible to predict how topographic maps are altered in complex genetic neurological diseases by analyzing ZebrinII molecular coding in postmortem tissue (R. V. Sillitoe and R. Hawkes, unpublished observation). Our study thus reveals a viable approach for retrospectively characterizing possible circuitry defects underlying human diseases involving the Cb.

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