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Embryonic origins of ZebrinII parasagittal stripes and establishment of topographic Purkinje cell projections

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Abstract

The establishment of neural circuits involves both the precise positioning of cells within brain regions and projection of axons to specific target cells. In the cerebellum (Cb), the medial-lateral (M-L) and anterior-posterior (A-P) position of each Purkinje cell (PC) and the topography of its axon can be defined with respect to two coordinate systems within the Cb; one based on the pattern of lobules and the other on PC gene expression in parasagittal clusters in the embryo (e.g. *Pcp2*) and stripes in the adult (e.g. *ZebrinII*). The relationship between the embryonic clusters of molecularly defined PCs and particular adult PC stripes is not clear. Using a mouse genetic inducible fate mapping (GIFM) approach and a *Pcp2-CreER-IRES-hAP* transgene, we marked three bilateral clusters of PC clusters with myristolated Green fluorescent protein (mGfp) on approximately embryonic day (E) 15 and followed their fate into adulthood. We found that these three clusters contributed specifically to *ZebrinII*-expressing PCs, including nine of the adult stripes. This result suggests that embryonic PCs maintain a particular molecular identity, and that each embryonic cluster can contribute PCs to more than one adult M-L stripe. Each PC projects a primary axon to one of the deep cerebellar nuclei (DCN) or the vestibular nuclei in the brainstem in an organized fashion that relates to the position of the PCs along the M-L axis. We characterized when PC axons from the three M-L clusters acquire topographic projections. Using a combination of GIFM to mark the PC clusters with mGfp and staining for human placental alkaline phosphatase (hAP) in *Pcp2-CreER-IRES-hAP* transgenic embryos we found that axons from each embryonic PCs cluster intermingled with neurons within particular DCN or projected out of the Cb toward the vestibular nuclei by E14.5. These studies show that PC molecular patterning, efferent circuitry, and DCN nucleogenesis occur simultaneously, suggesting a link between these processes.

Keywords

L7/Pcp-2; *ZebrinII*; molecular code; patterning; axon topography; deep nuclei

Introduction

The mammalian cerebellum (Cb) develops from a complicated series of cell migrations that eventually generate a structure with an exquisitely patterned morphology and micro-circuitry. At the gross anatomical level, fissures separate the Cb along the anterior-posterior (AP) axis

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into five cardinal lobes in the embryo (Altman and Bayer, 1996) and into ten lobules in the adult (Larsell, 1952). In the medial-lateral (M-L) axis three broad domains can be distinguished by morphology: a medial vermis that is surrounded by two hemispheres and more laterally by the flocculi/paraflocculi each with distinct foliation patterns. Thus, morphology provides one coordinate system of positional information in the Cb. A second coordinate system is revealed by distinct embryonic and adult gene expression patterns. Importantly, stripes of Purkinje cell (PC) gene expression are coincident with sensory-evoked Cb stripes (Chockkan and Hawkes, 1994; Hallem et al., 1999), and physiological manipulation of a particular stripe can result in distinct behavioral abnormalities (Attwell et al., 1999). Given the likely functional importance of Cb stripes, we set out to determine whether a link exists between the embryonic pattern of PC clusters and the adult pattern of PC stripes.

As early as E14, clusters of PCs along the M-L axis transiently express a number of markers including, *Purkinje cell protein-2* (*Pcp2*, Vandaele et al., 1991), also known as *L7* (Oberdick et al., 1993), the homeobox genes *Engrailed-1* and *-2* (Millen et al., 1995), and Calbindin (Wassef et al., 1985; reviewed by Larouche and Hawkes, 2006 and Sillitoe and Joyner, 2007). The most extensively studied marker of adult M-L stripes is ZebrinII/Aldolase C (Brochu et al., 1990; Ahn et al., 1994). Unlike the transient embryonic expression of genes in PC clusters, Tano et al. (1992) demonstrated in mice that ZebrinII is initially expressed in all PCs of the posterior lobules of the vermis at P5 and spreads gradually to all lobules by P12 (initially described in rats by Leclerc et al. (1988)). By P15, ZebrinII expression is suppressed in a subset of PCs resulting in the formation of parasagittal stripes selectively in lobules I-V and VIII-IX. Another adult M-L marker, Hsp25 (25 kDa heat shock protein) is instead expressed selectively in PC stripes in lobules VI/VII and IX/X (Armstrong et al., 2000). As for ZebrinII, the formation of the Hsp25 stripe pattern is complex. Stripes of Hsp25 expression are obvious in the vermis of the anterior lobules by P0 and PCs expressing Hsp25 are scattered in lobule VIII. Between ~P6–P9 all PCs in the Cb express Hsp25. Similar to ZebrinII the adult pattern is achieved through repression of Hsp25 in a distinct subset of PCs (Armstrong et al., 2001). Because the pattern of molecular coding is not the same in all lobules, it has been used to divide the vermis into four broad transverse zones: the anterior zone (AZ: lobules I-V), the central zone (CZ: lobules VI, VII), the posterior zone (PZ: lobules VIII, anterior (a) IX), and the nodular zone (NZ: lobules posterior (p) IX, X) (Ozol et al., 1999). We have suggested that the A-P and M-L organization of the lobules and molecular code (ML gene expression) underlie a developmentally significant two-coordinate system that both defines the three dimensions of the Cb and reflects positional cues used to pattern the Cb (Sillitoe and Joyner, 2007).

Three studies have provided indirect evidence that the embryonic PC clusters might have a lineage relationship to adult PC stripes (Ozol et al., 1999; Larouche et al., 2006; Marzban et al., 2007). The expression of Phospholipase C beta 4 (*Plcβ4*), which has a complementary pattern to ZebrinII in the adult (Sarna et al., 2006), is expressed in stripes from around birth through to the adult (Marzban et al., 2007), raising the possibility of a conservation of PC molecular identity during development. However, since early postnatal PCs that express *Plcβ4* and another gene, *Neurogranin* (Larouche and Hawkes, 2006), could be different from those expressing them in the adult, it is not certain whether specific embryonic PC subsets are related to each of the PC stripes in the adult. The situation is especially complicated since the number of stripes in the embryo is apparently less than in the adult (Larouche and Hawkes, 2006). Lineage studies using retroviruses reported that PCs maintain their general M-L position throughout development (Lin and Cepko, 1999; Mathis et al., 1997). Furthermore, a viral labeling approach showed that PCs born on a specific day occupy distinct parasagittal stripes in the adult mouse Cb (Hashimoto and Mikoshiba, 2003). None of these studies, however, has successfully followed the lineage of specific embryonic PC clusters and linked them to adult PC stripes.

In the adult Cb there exists a broad M-L efferent topography that connects PCs to the deep cerebellar nuclei (DCN) and vestibular nuclei in the hindbrain. PCs in the vermis project primarily to the fastigial nuclei (most medial DCN) and vestibular nuclei, PCs in the paravermis project to the interposed nuclei, and PCs in the hemispheres project to the most lateral dentate nuclei (reviewed by Sillitoe and Joyner, 2007). A previous *in vitro* tracing study by Eisenman et al. (1991) demonstrated using horse radish peroxidase (HRP) that PCs in rat Cb slices project to their target cells within the DCN and vestibular nuclei by ~E18 (corresponds to ~E16 in mouse). However, it is unclear how the projections from molecularly distinct PC clusters relate to their target DCN. Furthermore, since when HRP is used to mark cells in embryonic slice cultures labeled cells and their axons can only be followed for a short time before the slice integrity declines, it is unclear how each embryonic M-L cluster and its efferent projections ultimately relates to the adult M-L PC stripes.

Here we have used genetic inducible fate mapping (GIFM) (Joyner and Zervas, 2006) to address whether embryonic PCs expressing a *Pcp-2* transgene within specific clusters acquire reproducible M-L locations and have a defined fate with respect to adult ZebrinII-expressing PCs. By genetically marking a subset of terminally differentiated PCs and their axons with myristolated Green fluorescent protein (mGfp) based on their expression of *Pcp2-CreER-IRES-hAP* transgene and a reporter allele between ~E14.5–E15.5, we were able to follow the movement of three specific PC clusters during development. We have determined that M-L positional information is indeed conserved during the morphogenesis from nascent PC clusters to adult M-L stripes. We have also found that *Pcp2-CreER-IRES-hAP*-expressing PCs project their axons into specific target fields soon after they are born.

Experimental Procedures

Mouse strains

A *Pcp2-CreER-IRES-hAP* transgene was constructed by PCR subcloning an ~3kb fragment of *Pcp2* genomic sequence from the *Pcp2*ΔAUG/1B plasmid (gift from Dr. John Oberdick) containing 0.5kb of 5' sequences into a shuttle vector, and subcloning CreERT2 (Feil et al., 1997; gift from Dr. Pierre Chambon) into a vector containing an *IRES* sequence followed by a human placental Alkaline phosphatase cassette. *CreER-IRES-hAP* was then ligated into the fourth exon of the plasmid containing the *Pcp2* truncated enhancer and gene. Purified *Pcp2-CreER-IRES-hAP* transgene was then injected into the pronuclei of FVB/N fertilized mouse eggs (Nagy et al., 2003) by the NYUSoM Transgenic Core. Four founder lines were identified by PCR with primers for *CreERT2* (primer 1, 5'-GCCTGGTCTGGACACAGTGCC-3'; primer 2, 5'-CTGTCTGCCAGGTTGGTCAGTAAGC-3'; band size 397 bp). *Pcp2-CreER-IRES-hAP* transgenic lines were maintained on an outbred Swiss Webster background. The line with the strongest hAP expression in embryonic PC clusters and adult M-L PC stripes was chosen for all subsequent studies. *Pcp2-CreER-IRES-hAP* transgenic mice were bred with *Tau^{lox-STOP-lox-myristolatedGfp-IRES-NLS-LacZ}* knock-in (*Tau^{mGfp}*) reporter mice (gift of Dr. Silvia Arber; Hippenmeyer et al., 2005), and animals carrying both alleles were used for fate mapping experiments. *Tau^{mGfp}* transgenic mice were genotyped using Gfp primers (primer 1, 5'-CTGGTCGAGCTGGACGCGACG-3'; primer 2, 5'-CACGAAGTCCAGCAGGACCATG-3'; band size 650 bp). In order to activate *CreERT2* and induce recombination of the *loxP* sites, tamoxifen (Tm, Sigma, St. Louis, MS) in corn oil was administered by oral gavage at noon on E14.5 at 4 mg/40 g mouse. The day a vaginal plug was detected was designated E0.5 and E19 was considered the day of birth or postnatal (P) day 0.

Perfusion and immunohistochemistry

Adult mice were transcardially perfused with 4% paraformaldehyde (PFA) and analyzed by immunohistochemistry as described previously (Sillitoe et al., 2008).

Antibodies

Antibodies were used as described previously (Sillitoe et al., 2008) or from: Molecular Probes rabbit polyclonal anti-GFP (1:750; Carlsbad, CA), Rockland Immunochemicals Inc. goat polyclonal anti-GFP (1:250; Gilbertsville, PA), Chemicon rabbit anti-Tbr1 (1:1000; Temecula, CA), Chemicon goat anti-Brn2 (1:1000), Biogenesis goat anti- β -gal (1:500; Brentwood, NH). Alexa-488 and Alexa-555 secondary antibodies (1:1500; Molecular Probes) were used as previously described (Zervas et al., 2004; Sillitoe et al., 2008). Nuclear fast red stained tissue was dehydrated in an ethanol series and mounted with DPX (Electron Microscopy Sciences, Hatfield, PA).

Detection of β -galactosidase and human Alkaline Phosphatase activity

Tissue sections were processed for X-gal histochemistry as described by Zervas et al. (2004) and for hAP staining as described by Badea et al. (2003). Whole mount Cb were cleared in 1:2 benzyl alcohol: benzyl benzoate after hAP staining at E14.5.

Image capture and analysis

Photomicrographs of tissue sections were captured using a Retiga SRV camera mounted on a Leica DM6000 microscope running Volocity software. Photomicrographs of whole mount stained Cb were captured using a Leica MZ16 FA stereomicroscope mounted with a Leica DFC300 FX camera running Leica LAS software (Montage module). The images were imported into Adobe Photoshop and corrected for brightness and contrast only.

Results

A *Pcp2-CreER-IRES-hAP* transgene reveals M-L organization of PCs in the developing and adult Cb

Pcp2 is expressed exclusively in Cb PCs and retinal bipolar neurons (Oberdick et al., 1988). Within the Cb *Pcp2* is initially expressed at E14.5 and only in parasagittal clusters of PCs (Oberdick et al., 1993), whereas after ~P9 it is expressed in all PCs (Smeyne et al., 1991, Oberdick et al., 1993; also see mRNA expression in the Allen Brain Atlas). The regulatory sequences 1kb 5' to the *Pcp2* promoter and within the introns have been shown to be sufficient to drive LacZ expression similar to endogenous *Pcp2* expression (Oberdick et al., 1993), whereas truncation of the 5' sequence to ~0.5kb results in parasagittal expression throughout development and in the adult (Oberdick et al., 1993). As a means to mark some of the PCs within the *Pcp2* parasagittal clusters in the embryo and follow them into the adult Cb, we generated transgenic mice that express an inducible form of the Cre recombinase (CreERT2) under the control of the 0.5kb 5' truncated *Pcp2* transgene (Oberdick et al., 1993). The Cre protein is fused to a mutated form of the estrogen-binding domain of the human Estrogen Receptor (CreER), to ensure specific and transient activation of Cre by Tm. Cre translocates into the nucleus when bound by Tm and then induces recombination of *LoxP* sites within engineered reporter alleles. Nuclear translocation of CreER occurs within 6 hours of Tm treatment and is then sustained for about 24 hours (Hayashi and McMahon, 2002; Zervas et al., 2004). In order to mark the axons of cells expressing the *Pcp2* transgene, an *IRES-hAP* (internal ribosomal entry site-human placental alkaline Phosphatase) cassette was included downstream of *CreER* in the transgene (*Pcp2-CreER-IRES-hAP*). The *Pcp2* promoter drives transcription of a mRNA containing both CreER and hAP, and the IRES sequence located between the two reporter gene sequences ensures independent but coordinate translation of the two proteins. Expression of the *Pcp2-CreER-IRES-hAP* transgene was characterized by analyzing hAP expression (n = 6 for each age). No hAP activity was detected at E13.5 (Fig. 1A). At E14.5, hAP activity was detected only in two clusters of PCs on either side of the midline in the posterior Cb (Fig. 1B). By E15.5, expression of the *Pcp2-CreER-IRES-hAP*

transgene in the two PC clusters was extended anteriorly and posteriorly (Fig. 1C, D). An additional two anterior clusters of hAP-expressing PCs were also detected more laterally on each side of the Cb (Fig. 1C, D). Between E15.5 and E16.5 the basic pattern remained the same, although more PCs within each cluster expressed hAP (Fig. 1D1–D3). By E18.5 transgene expression marked five major stripes of PCs that spanned the M-L axis and extended along the A-P axis to differing degrees. During postnatal development the transgene expression continued to mark 5 major M-L stripes (Fig. 1F–1H). Although between E18.5 and P8 the pattern of transgene expressing stripes remained similar (compare Fig. 1E and 1G), the hAP staining intensity in particular stripes appeared to decrease (for example see arrowheads in Fig 1E, 1F, 1G). This could be due to the rapid growth and expansion of the postnatal Cb and the resulting spread of PCs into a monolayer. M-L stripes of hAP activity were less clear in the adult (Fig. 1H). In the vermis at all ages, the CZ (lobules VI/VII) and PZ (lobules VIII/aIX) had the highest number of marked PCs, followed by the AZ and the NZ with the least number of labeled PCs. A literature-based comparison of our transgenic with a previously generated *Pcp2-LacZ* transgenic line containing a truncated 0.5kb enhancer (Oberdick et al., 1993) suggests some differences between the two. For example, whereas after E15.5 in our transgenic mice prominent clusters of transgene expression were detected in the AZ and in lobule IX, in the *Pcp2-LacZ* line the clusters in the anterior lobules were weak and lobule IX uniformly expressed the *Pcp2-LacZ*. It is likely that the variation in expression patterns observed between the two transgenic lines are due to differences in their sites of integration within the genome.

PCs migrate and settle within particular A-P and M-L locations in the Cb

In order to mark and follow the fate of *Pcp2-CreER-IRES-hAP*-expressing PCs, we crossed our transgene onto a *Tau^{mGfp}* reporter line (Hippenmeyer et al., 2005; see experimental procedures for details of the construct). Cre-mediated recombination results in permanent expression of myristolated Green fluorescent protein (mGfp) and nuclear β -galactosidase (β -gal). Only postmitotic neurons express *Tau* and therefore are the only cells labeled with mGfp and β -gal. To establish the relationship between the three pairs of parasagittal clusters of PCs that initially express *Pcp2-CreER-IRES-hAP*, we administered Tm to *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* embryos on E14.5. As CreER is active for ~36hr beginning 6hr after Tm administration, PCs expressing *Pcp2-CreER-IRES-hAP* between ~ E14.75–E16 should be marked. Our first step toward constructing a genetic fate map of the Cb molecular code was to analyze the initial marked population of PCs 24hr after Tm administration. At E15.5 only a few mGfp labeled cells were detected in the anterior Cb (the presumptive anterobasal and anterodorsal cardinal lobes) with many more marked cells consistently found in the posterior medial Cb (mainly within the presumptive central and posterior cardinal lobes) ($n = 12$; Fig. 2). We also found a large number of PCs within the Cb core (arrow Fig. 2D), which presumably were PCs migrating toward the periphery of the Cb. To confirm that only PCs were marked in *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* embryos treated with Tm on E14.5, we double-labeled tissue sections from E15.5 and E18.5 Cb with antibodies against ROR α (an embryonic PC marker; Hamilton et al., 1996; Maricich and Herrup, 1999; Ino, 2004; Suppl Fig. 1, Suppl Fig. 2) and mGfp. Co-labeling of ROR α with mGfp showed that indeed at both ages analyzed all mGfp-marked cells are immunoreactive for ROR α (Fig. 3).

Analysis of *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* embryos treated with Tm at E14.5 and analyzed at E18.5 uncovered that the marked PCs in the vermis were restricted to particular lobes in the A-P axis ($n = 23$) and most cells populated the PC layer (Fig. 2F–2I). Compared to E15.5, more marked PCs appeared to be present at E18.5, based on mGfp and β -gal expression, likely due to the longer exposure to Tm and the subsequent increase in the number of PCs undergoing CreER-mediated recombination and having time to express mGfp and LacZ (Fig. 2F–2J). We observed a few marked PCs in some sections of the anterodorsal lobe (red asterisk in Fig. 2H) and many marked cells in most sections of the central and posterior cardinal lobes at E18.5

(Fig. 2F–2I). Only a few marked cells were found in the anterobasal (not shown) and inferior cardinal lobes (red asterisk in Fig. 2I). Note that within the central lobe, however, PCs at E18.5 were more scattered than in other lobes (arrows, Fig 2F and 2G), consistent with the late cytoarchitectural development in this lobe (Altman and Bayer, 1996). In more lateral regions of the Cb (presumptive hemispheres) labeled PCs were restricted along the A-P axis to the central region.

We followed the three pairs of marked PCs clusters in *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* embryos treated with Tm at E14.5 into the adult Cb (n = 17), and found that similar to E18.5 these PCs still primarily populated the CZ and PZ of the vermis, which are derived from the central and posterior cardinal lobes (Fig. 4A, 4B). Double immunostaining for mGfp and the PC specific marker Calbindin confirmed that as in the embryo, all marked cells could be molecularly (Fig. 4C) and anatomically identified as being PCs (Fig. 4D, Fig. 4H). In the AZ (lobules I–V, Fig. 4A) marked cells were mainly seen in lobule IV/V (arrows Fig. 4A), which is derived from the posterior anterodorsal cardinal lobe. Only a few marked cells were found in the remainder of the AZ and NZ (arrows Fig. 4B). In the hemispheres marked PCs were found in CrusI, CrusII, and the paramedian lobule (data not shown and see Fig. 8).

In the M-L axis PCs of *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* embryos marked by Tm treatment at E14.5 contributed to three pairs of M-L PC clusters at E15.5 (Fig. 5A), E16.5 (Fig. 5B–5C) and E18.5 (Fig. 5D and data not shown). Furthermore, the medially located pair of clusters at both ages was most heavily labeled (labeled as #1 in Fig. 5). Conversely, the two lateral pairs of clusters had only a few marked cells in each section (e.g. Fig. 5A). These data are consistent with the initial expression of *Pcp2-CreER-IRES-hAP* in three pairs of M-L PC clusters with highest levels in the medial clusters. As in the embryo, the adult stripes closest to the midline were most obvious (Fig. 5E). mGfp and β -gal expression at E18.5 and in the adult showed that Tm treatment at E14.5 in *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* embryos only marked cells that were morphologically identified as being PCs (Fig. 5F). Thus, our data show that PCs expressing *Pcp2-CreER-IRES-hAP* during ~E14.75–E16 are located in specific A-P and M-L locations at E15.5, and although they continued to migrate after E15.5, we infer that movement within the M-L axis is minimal since the locations of the three M-L clusters at E18.5 are similar to at E15.5.

***Pcp2-CreER-IRES-hAP* embryonic parasagittal PC clusters have a specific relationship to the adult molecular code**

We next asked whether the fate mapped PCs in *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* embryos were restricted to the ZebrinII positive or negative cells (Brochu et al., 1990; Sillitoe and Hawkes, 2002).

Vermis—Based on the pattern of mGfp staining alone, no obvious relationship between the fate-mapped cells and the striped pattern of ZebrinII could be detected. Strikingly however, within the AZ (Fig. 6A–6D) and PZ (Fig. 6E–6G) we found that virtually all the fate-mapped PCs were double-labeled with mGfp and ZebrinII (arrows Fig. 6). We found ~50 mGfp marked PCs on each tissue section cut through the PZ, and of these only 1–2 PCs were located within the ZebrinII negative stripes and expressed mGfp alone (arrowheads Fig. 6F, 6H, 6I). Furthermore, in the PZ the fate mapped PCs were located almost exclusively within the ZebrinII P2+ stripes (Fig. 6E, 6F), although some marked PCs were also found in the PZ ZebrinII stripes P1+, P3+, and P4+ (Fig. 6F, 6G). ZebrinII is expressed in a variable set of stripes in lobule VIa (Fig. 6A), a “transition zone”, and in all PCs in lobule VIb and VIIa of the CZ (Fig. 6F) (Ozol et al., 1999). The fate-mapped cells in the CZ were all ZebrinII immunoreactive as ZebrinII is expressed throughout this region (e.g. Fig. 6E). The fate-mapped PCs were scattered and had no obvious M-L pattern in the CZ. Accordingly, we found no correlation between mGfp marked

cells and the pattern of Hsp25 in the CZ (Suppl Fig. 2). To further test whether all fate-mapped PCs were located only within ZebrinII⁺ stripes in lobule VIII we double-labeled PCs with mGfp and Plcβ4 that selectively labels the ZebrinII negative PCs (Sarna et al., 2006; Fig. 7A, 7B). Consistent with the ZebrinII staining, Gfp/Plcβ4 double staining revealed that the vast majority of PCs expressed either mGfp or Plcβ4 (Fig. 7), with only rare PCs co-expressing these two proteins (blue arrows in Fig. 7F–7H).

Unlike ZebrinII that has a spatially dynamic expression profile during the first two postnatal weeks, Plcβ4 is thought to mark the same set of parasagittal stripes of PCs from late embryonic ages through adulthood when it is found in all ZebrinII negative Purkinje cells (Marzban et al., 2007). Accordingly, in adult *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* mice administered Tm at E14.5 Plcβ4-expressing PCs did not co-express mGfp (Fig. 7A–7H). We next tested whether the cells that express Plcβ4 in the early postnatal cerebellum maintain expression into the adult by double labeling the cerebellum of P2 *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* mice treated with Tm at E14.5 for mGfp and Plcβ4. Indeed, we observed that most mGfp labeled PCs occupied the Plcβ4[−] stripes, and only found rare cells co-expressing mGfp and Plcβ4 within the Plcβ4⁺ stripes (n = 4; Fig. 7I). Furthermore, while mice treated with Tm at E14.5 and analyzed at P5 showed some mGfp⁺ cells located within the Plcβ4 “positive” stripes, in most cases the cells did not co-express mGfp and Plcβ4 (Fig. 7J, 7K). Thus, the PCs become lineage restricted as Plcβ4⁺ or ZebrinII⁺ subsets at least as early as P2, and likely by ~E15.5 when cells are first marked.

Hemispheres—In mice six ZebrinII⁺ stripes (P4a, P4b, P5a, P5b, P6 and P7) occupy three lobules in the hemispheres, CrusI, CrusII and the paramedian lobule (see Sillitoe and Hawkes, 2002 for nomenclature). After marking PCs at ~E15.5 we detected mGfp⁺ cells in all six ZebrinII stripes of the adult hemispheres. However, more marked cells were located in P4a⁺, P4b⁺, P5a⁺ and P5b⁺ than in P6⁺ and P7⁺ (Fig. 8). We also detected scattered mGfp⁺ cells in the lateral-most extent of the copula pyramidis (Fig. 8) and in the lobulus simplex (data not shown). Importantly, as in the vermis, most Gfp⁺ marked PCs in the hemispheres were ZebrinII⁺ (Fig. 8). These data indicate that PCs within the three *Pcp2-CreER-IRES-hAP*-expressing embryonic clusters contribute primarily to four ZebrinII⁺ stripes in the hemisphere, one ZebrinII⁺ stripe in the PZ of the vermis, as well as to the CZ where ZebrinII is expressed homogeneously.

The topography of PC-target organization is established early during embryonic Cb development

We next visualized axon morphology by genetically labeling PCs with mGfp and hAP expression to address the extent to which the topographical order of PC-targets is established during embryonic development. Based on the *in vitro* study of rat Cb slices by Eisenman et al. (1991), mouse PCs might project to the DCN and vestibular nuclei by ~E16 *in vivo*.

PC to DCN projections—Within each sub-nucleus of the DCN, each PC axon can simultaneously contact small, gamma-aminobutyric acid (GABAergic) inhibitory interneurons and large, glutamatergic excitatory projection neurons (De Zeeuw and Berrebi, 1995). To characterize PC axon-target invasion *in vivo*, we visualized mGfp⁺ PCs in *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* embryos treated with Tm on E14.5 and hAP⁺ PC axons in *Pcp2-CreER-IRES-hAP* embryos as they projected into their target fields within the DCN and vestibular nuclei.

By E18.5 we found that in *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* mice each M-L PC cluster projected distinct bundles of axons within straight trajectories toward the DCN (Fig. 9B–9D, see Fig. 10 for DCN sub-nuclei). Consistent with *Pcp2-CreER-IRES-hAP* expression being most prominent in the vermis (Fig. 1), the densest labeling of mGfp⁺ axons was in the fastigial

nucleus. At E18.5 we found many labeled axons within all three DCN (Fig. 9). Based on serial section and whole mount hAP analyses our assessment is that each PC cluster projects axons to more than one DCN sub-nucleus, although each one has a major path related to its M-L position (e.g. the most medial cluster projected mainly to the fastigial nucleus; Fig. 9F). Similar patterns were observed as early as E15.5 and E16.5 (data not shown and see Fig. 9A and Fig. 11).

In order to confirm that PC axons were indeed extending into their target domains within the DCN, we used the expression of T-box brain1 (*Tbr1*; Fink et al., 2006) that labels the fastigial population of glutamatergic excitatory projection neurons, and Brain-2 (*Brn2*) that marks a subset of the interposed and dentate neurons (Fink et al., 2006; Fig. 10). At E15.5 PC axons were found within the two *Brn2* populations (Fig. 11A, 11B) and PCs with varying orientations of their processes were located both within the DCN (Fig. 11B) and in the developing PC layer (inset Fig. 11B). At E18.5 the medial PC cluster terminated mainly with the fastigial nucleus and not in the *Brn2* expressing interposed and dentate nuclei. Cluster #3 did terminate within these latter two nuclei (inset Fig. 11C). In the adult Cb mGfp stained axons were found mainly within the fastigial nucleus with additional staining in the interposed and dentate nuclei (Fig. 9E and data not shown).

PC to vestibular nuclei projections (the cerebello-vestibular tract)—In addition to projecting to the DCN, PCs also project to neurons in the vestibular nuclei of the ventral hindbrain (Voogd et al., 1991). This pathway forms the cerebello-vestibular tract that is crucial for maintaining balance during motion (Yakusheva et al., 2007). While some axons appeared to terminate on the *Tbr1* expressing cells at E18.5 (Fig. 11F, 11G), many mGfp+ axons clearly passed through the fastigial nucleus en route to the vestibular nuclei via the inferior cerebellar peduncle (thick arrows Fig. 11C, 11E). We next examined hAP expression from the *Pcp2-CreER-IRES-hAP* transgene in order to analyze PC axons at E14.5 when the transgene is first expressed during development. Strikingly, in whole mount hAP-stained Cb we observed a thick bundle of PC axons emerging from the one medial pair of bilaterally marked PC clusters as early as E14.5, and could trace these axons through the inferior cerebellar peduncles (see white arrows Fig. 1B). In order to follow single axons into the brainstem we analyzed *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* embryos treated with Tm at E14.5 for mGfp expression. While only a few mGfp+ axons were seen in the brainstem at E15.5 (data not shown), at E16.5 (n = 13) a dense bundle of PC axons was detected exiting the inferior cerebellar peduncles and entering the brainstem (arrow in Fig. 9A). By E18.5 the cerebello-vestibular projection was robust and mGfp+ axons were found throughout the vestibular nuclei (Fig. 9B–9D). Furthermore, double labeling of mGfp and *Tbr1*, which also marks a subset of vestibular nuclear neurons (Wang et al., 2005), confirmed that PC axons intermingle with neurons of the vestibular nucleus by E18.5 (Fig. 11D, 11E) and also in the adult (Fig. 9E). These results indicate that PC axons from the vermis that form the cerebello-vestibular tract have fasciculated and exited the Cb by ~E14.5, an age earlier than previously appreciated. Furthermore, our data indicate that the cerebello-vestibular tract axons have infiltrated the vestibular nuclei by at least E16.5.

Discussion

Embryonic parasagittal clusters have a defined lineage within the adult parasagittal stripes

Previous gene expression studies have predicted a correlation between the embryonic M-L molecular code and M-L stripes in the adult Cb (Ozol et al., 1999; Larouche et al., 2006; Marzban et al., 2007). Our fate-mapping experiments demonstrate that at least for the PCs marked in *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* embryos treated with Tm at E14.5, the embryonic M-L molecular code does indeed carry patterning information forth to the adult M-

L molecular code. We found that *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* PCs marked at ~E15.5 take on the distinct phenotype of being ZebrinII⁺. Furthermore, the marked PCs at both P2 and in the adult are Plcβ4[−] indicating that indeed cells maintain their Plcβ4 expression profile throughout development. These observations support the argument that the molecular lineage of specific PC subsets and their ultimate locations within specific M-L stripes is determined on or soon after their birth.

In the PZ the majority of fate mapped PCs were located within the P2+ ZebrinII stripe. A previous comparison of *Pcp2-lacZ* transgene expression to ZebrinII stripes using a line containing the same 0.5kb *Pcp2* enhancer also showed predominant co-localization of X-gal and ZebrinII within P2+ in lobule VIII and only a few co-labeled cells in P1+ of lobule IX (Ozol et al., 1999). However, Ozol et al. (1999) found many X-gal labeled cells in the ZebrinII[−] stripes of the AZ. This result is nevertheless consistent with the anterior expansion of hAP expression we detected after E16.5 in our transgene (see Fig. 1), and marking of many PCs in the ZebrinII[−] stripes of the AZ when Tm was given at E17.5 (data not shown).

In terms of A-P positioning of PCs, it has been assumed that each of the five cardinal lobes in the embryo gives rise to specific lobules in the adult Cb (Altman and Bayer, 1997). Our fate-mapping data reveals that indeed PCs within the central lobes in the embryo are restricted during development to be located within lobules VI-VIII in the adult. This pattern of settlement indicates that once PCs reach the putative PC layer, their movement is restricted within both the A-P and M-L axes.

Each PC embryonic cluster disperses into multiple parasagittal stripes in the adult Cb

Previous marker analyses using Neurogranin and Plcβ4 expression, markers that bridge between embryonic clusters and adult stripes, have suggested that embryonic PC clusters can give rise to multiple adult stripes (Larouche et al., 2006; Marzban et al., 2007). In addition, a retrospective clonal analysis of Cb VZ cells using retroviruses demonstrated that single clones can contribute to multiple stripes (Lin and Cepko, 1999). By marking specific clusters of *Pcp2-CreER-IRES-hAP*-expressing PCs and following their fate into the adult Cb, our data provide compelling evidence that each embryonic cluster does indeed give rise to multiple stripes in the adult (Fig. 6 and Fig. 8).

Topography of the PC-target organization is established early during embryonic Cb development

Cb neurons are generated according to a well-defined sequence of neurogenesis from two specialized germinal zones: the rhombic lip (RL) and the VZ. PCs are derived from the VZ and become postmitotic between E11 and E13 (Miale and Sidman, 1961) and thereafter migrate radially into the Cb core using glial processes as migratory guides (Morales and Hatten, 2006). The DCN neurons that are derived from both the RL (Machold and Fishell, 2005; Wang et al., 2005; Fink et al., 2006) and VZ (Hoshino et al., 2005) are generated between ~E10 and E13. Our marker analysis reveals that interactions between the axons of migrating PCs and the DCN are likely in place by E15.5 (Fig. 11), indicating that the cues necessary for Cb efferent circuit topography are established early during Cb embryogenesis. Thus, some of the genes with M-L PC expression patterns in the embryo before E15.5 might determine the functional circuit organization of the adult Cb.

Furthermore, the results of our experiments indicate that PC axonogenesis must begin soon after, or on the day of their birth (between E11–E14) to accomplish the long trajectories we observed in *Pcp2-CreER-IRES-hAP* PCs at E14.5, including axons extending into specific DCN and into the peduncles. Moreover, as PCs and DCN neurons are still migrating at E15.5,

the initial topography of the circuit is established well before the somata of either neuron type in the circuit has settled.

A perspective of topographic circuit formation in the cerebellum

Although recent developments in physiology and genetics have advanced our knowledge of circuit function in the awake behaving adult animal (Adamantidis et al., 2007), we know much less about how these circuits are wired together during development and whether embryonic interactions between neurons are critical for the functional circuitry. Our data sheds new light upon this problem by providing evidence that distinct PC lineages interact with their target cells soon after their birth, and that there is a M-L conservation of connectivity between PC clusters and the DCN into adulthood. Furthermore, the early interactions mirror the core circuitry that will later incorporate additional synaptic input from long distance afferent sources and local interneurons. By extrapolating from our data, soon after PCs are born there must be a simultaneous rapid extension of their axons toward target sources and migration of the cell body, which produces a skeleton of the ultimate efferent circuitry. It will be interesting to determine whether the initial Cb circuits exhibit any spontaneous activity, and whether activity is necessary for further refinement of the circuitry. Since we saw only limited A-P and M-L movement of PCs, and since mossy and climbing fiber afferent sources terminate within specific M-L bands in distinct lobules (reviewed by Larouche and Hawkes, 2006; Sillitoe and Joyner, 2007), it is possible that each embryonic cluster produces factors that attract or repel select afferent axons. The transformation of embryonic circuitry to the functional adult circuits likely involves a much greater degree of complexity than dispersion of PCs. Our future studies are aimed at deciphering precisely how afferent and efferent map topography is genetically regulated, in part by identifying the molecular signals required for cell-to-cell communication during Cb circuit formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. hAP staining in *Pcp2-CreER-IRES-hAP* mice reveals PC stripes throughout development
A, No staining was observed at E13.5 (dorsal view). **B**, By E14.5 (posterior-inferior view) two distinct M-L clusters were seen on each side of the Cb (black arrows). **C**, By E15.5 (dorsal view) there are an additional two PC clusters (numbered as 2, 3). **D**, Anterior view of an E15.5 Cb showing weaker staining in the anterior Cb (below the black horizontal line and black arrow). **D1–D3**, Tissue sections stained for hAP at E16.5. Numbers indicate PC clusters and asterisks indicate weak staining. In the dorsal view schematic (**D3**) the black vertical lines indicate the level of where the sagittal tissue sections in **D1–D3** were taken from. **E**, **F**, Between E18.5 to P3 expression within each cluster intensifies and expands anteriorly (asterisks in **E**) and posteriorly. **G**, M-L stripes were seen By P8. Lobules VI–VII were uniformly stained. **H**, Stripe boundaries were poorly delineated in the adult. Lobules are indicated by Roman numerals (applies to all figures). Abbreviations: Bs, brainstem; Cb, cerebellum; Cp, choroid plexus; Ctx, cerebral cortex; icp, inferior cerebellar peduncle; Mb, midbrain; A, anterior; P, posterior. Scale bar in **C** = 1mm (also applies to **A**), **D** = 500 μ m (also applies to **B**), **D3** = 250 μ m, **E** = 500 μ m, **F** = 1 mm, **H** = 1 mm.

Figure 2. *Pcp2* expressing PCs are located within specific A-P locations during embryogenesis A–J. Mice treated with tamoxifen at E14.5 and analyzed using mGfp on sagittal sections at E15.5 and E18.5. By E18.5 most PCs were located at the periphery of the Cb cortex. A few were located internally (arrow in **G**; see also Fig. 3). The red asterisks in **H** and **I** indicate marked cells in the ADL and INL, respectively. Abbreviations: ABL, anterobasal lobe, ADL, anterodorsal lobe; CEL, central lobe; POS, posterior lobe; INL, inferior lobe; B-gal, B-galactosidase; Cb, cerebellum; cp, choroid plexus; ic, inferior colliculus; mGfp, myristolated Green fluorescent protein. Scale bar in **F** = 250 μ m (applies to all panels).

Figure 3. PCs are selectively marked in *Pcp2-CreER-IRES-hAP;Tau^{mGfp/+}* mice
A–H, Mice treated with tamoxifen at E14.5 and analyzed on sagittal sections at E15.5 and E18.5. mGfp expression (red) showing that all transgene expressing cells were ROR α positive (green) PCs. mGfp expression revealed PC somata and processes. The regions outline by boxes in **C** and **G** are shown at higher power in **D** and **H**. Abbreviations: egl, external granular layer; pcl, developing Purkinje cell layer, CEL, central lobe; POS, posterior lobe. The scale bar in **A** = 125 μ m (applies to **B**, **C**, **E**, **F**, **G**), **H** = 62.5 μ m (applies to **D**).

Figure 4. *Pcp2* expressing PCs preserve their A-P locations during development

A, B, Mice treated with tamoxifen at E14.5 and analyzed by mGfp expression (red) and Calbindin (green) on sagittal sections at P30. **C, D,** higher power magnification of marked PCs. The arrow in **D** points to an axon. Abbreviations: CZ, central zone; PZ, posterior zone; ml, molecular layer; pcl, Purkinje cell layer; gcl, granule cell layer. The scale bar in **B** = 1 mm (applies to **A**), **C** = 125 μ m, **D** = 50 μ m.

Figure 5. *Pcp2* expressing PCs are located within specific M-L clusters

A–F, Mice treated with tamoxifen at E14.5 and analyzed on coronal sections at E15.5, E16.5, E18.5, and P30. mGfp expression (red) showing that marked PCs were located mainly within three distinct M-L clusters. **B**, Whole mount staining for X-gal at E16.5. Clusters 1 and 2 have the highest number of marked PCs. **F**, mGfp and B-gal double stained PCs at P30.

Abbreviations: B-gal, B-galactosidase; H, hemisphere; hAP, human placental Alkaline phosphatase; mGfp, myristolated Green fluorescent protein. Scale bars in A = 250 μ m (applies to C, E, D, F), B = 500 μ m, G = 500 μ m, H = 50 μ m.

Figure 6. *Pcp2* expressing PCs are restricted to ZebrinII stripes in the vermis

A, Mice treated with tamoxifen at E14.5 and analyzed on coronal sections at P30 using mGfp expression (red) showing that marked PCs in the AZ co-labeled with ZebrinII (green). The area outlined by the box in **A** is shown at higher power in **B–C**. Marked PCs located within ZebrinII “negative” stripes expressed mGfp and ZebrinII (arrows in **B–D**). **E–H** illustrates the restriction of marked PCs to ZebrinII positive stripes in the PZ (three different animals). The higher power images in **I** and **J** were taken from different animals and illustrate the predominance of mGfp marked cells within ZebrinII positive stripes. Occasional mGfp positive/ZebrinII negative PCs were located within ZebrinII negative stripes (arrowheads in **F, H, I**). Scale bar in **A** = 500 μ m (applies to **E–H**), **D** = 250 μ m (applies to **B, C, I, J**).

Figure 7. *Pcp2* expressing PCs are located within complementary stripes to *Plcβ4*

A, E, ZebrinII and *Plcβ4* have complementary stripe patterns in the AZ (**A**) and PZ (**E**). Mice treated with tamoxifen at E14.5 and analyzed on coronal sections at P30 showing that mGfp (red) marked PCs are *Plcβ4* (green) negative in the AZ (**B–D**) and PZ (**F–H**). A few marked cells were found within the *Plcβ4* positive stripes (arrows in **B–C**) and some appeared to co-express mGfp and *Plcβ4* (blue arrows in **F–H**). At P2 (**I**) and P5 (**J, K**) most AZ PCs did not express *Plcβ4* (lateral arrows in **I** and right arrow in **K**). A few did co-express mGfp (middle arrow in **I**) or were closely adjacent to PCs expressing mGfp (left arrow in **K**). The area outline by the box in **J** is shown at higher power in **K**. Scale bar in **A** = 750 μ m (applies to **E**), **B** = 500 μ m (applies to **C, D, F, G, H**), **I** = 250 μ m (applies to **J, K**), **K** = 100 μ m.

Figure 8. *Pcp2* expressing PCs are restricted to ZebrinII stripes in the hemispheres
Mice treated with tamoxifen at E14.5 and analyzed on coronal sections at P30 using mGfp expression (red, **A**, **C**) showed that marked cells were co-labeled with ZebrinII (green, **B**, **C**). Arrows point to examples of double labeled PCs. **D–F** are higher power images of CrusII and the pmd illustrating the restriction of mGfp marked cells to ZebrinII positive stripes. Abbreviations: pmd, paramedian lobule; cop, copula pyramidis. Scale bar in **C** = 500 μ m (**A–C**) and 250 μ m (**D–F**).

Figure 9. PC axons are restricted to M-L locations in the Cb

Mice treated with tamoxifen at E14.5 and analyzed on coronal sections at E16.5 (**A**), E18.5 (**B–D**) and P30 (**E**) using mGfp expression (red). PCs clusters are labeled as 1–3. The DCN in **E** are outlined with dotted lines. **F**, Schematic illustrating axon contributions of each PC cluster to the DCN and VN. Thicker arrows represent larger contributions. Abbreviations: V4, fourth ventricle; icp, inferior cerebellar peduncle; ic, inferior colliculus; cp, choroid plexus; FN, fastigial nucleus; IN, interposed nucleus; DN, dentate nucleus; VN, vestibular nucleus; bv, blood vessel, m, midline of Cb. Scale bar in A = 250 μ m (applies to B, C), D = 125 μ m, E = 250 μ m.

Figure 10. Tbr1 is expressed in the fastigial nucleus and Brn2 is expressed in the interposed and dentate nuclei

Coronal cut sections through E15.5 (A), E16.5 (B), and E18.5 (C) Cb showing that Tbr1 (red) is mainly expressed in neurons of FN while Brn2 (green) is mainly expressed in neurons of the IN and DN. The E15.5 Cb is outlined with a dotted line. The arrow in A points to the ventricular zone. Abbreviations: V4, fourth ventricle; FN, fastigial nucleus; IN, interposed nucleus; DN, dentate nucleus; VN, vestibular nucleus, Bs, brainstem, m, midline of Cb. Scale bar in C = 100 μ m (applies to A, B).

Figure 11. *Pcp2* expressing PCs project axons to topographical locations within the DCN and vestibular nuclei

Mice were treated with tamoxifen at E14.5 and analyzed on coronal sections at E15.5 (**A**, **B**), and E18.5 (**C**–**G**) using mGfp expression (red) and either *Tbr1* or *Brn2*. By E15.5 mGfp marked PC somata (asterisk in **A**, arrows in **B**) and their axons (circled region in **B**) are intermingled with *Brn2* expressing neurons (green). Inset in **B**, The cluster of PCs labeled as #2 is from a near adjacent section compared to panel **A**. The area outlined by the box in **A** is shown at higher power in **B**. Abbreviations: V4, fourth ventricle; FN, fastigial nucleus; icp, inferior cerebellar peduncle; IN, interposed nucleus; DN, dentate nucleus; VN, vestibular nucleus; VZ, ventricular

zone; m, midline of Cb. Scale bar in C = 250 μm (applies to A and is = 500 μm for the inset in C), the inset in B = 50 μm , F = 100 μm (applies to B, D), G = 50 μm (applies to E).