Close Correlation Between the Birth Date of Purkinje Cells and the Longitudinal Compartmentalization of the Mouse Adult Cerebellum

Kazunori Namba,[1] Izumi Sugihara,[2] and Mitsuhiro Hashimoto[1]*

[1]Hashimoto Research Unit, RIKEN BSI, Saitama 351-0198, Japan
[2]Department of Systems Neurophysiology, Tokyo Medical and Dental University, Graduate School of Medicine, Tokyo 113-8519, Japan

ABSTRACT

The adult cerebellum is organized into longitudinal compartments that are revealed by specific axonal projections (olivocerebellar and corticonuclear projections). These compartments in the adult cerebellum are closely correlated with the striped expression of zebrin II (aldolase C), a late-onset marker of Purkinje cells. Similarly, the embryonic cerebellum is organized into longitudinal compartments that are revealed by striped expression of other genes (early-onset markers). The cerebellar compartments are thought to be the basic and functional subdivisions of the cerebellum. However, the relationship between the embryonic (early-onset) and the adult (late-onset) compartments has remained unknown, because the pattern of the embryonic compartments is distinct from that of the adult compartments. To examine this issue, we labeled Purkinje cells (PCs) born at embryonic day (E) 10.5, E11.5, and E12.5 by using an adenoviral vector and traced their fated positions in the adult cerebellum. By comparing the striped distribution of each cohort of birth date-related PCs with the striped pattern of zebrin II immunoreactivity (zebrin II bands) in the entire adult cerebellum, we found that the striped distribution of PCs correlated strikingly with zebrin II bands. Generally, a single early-onset compartment was transformed directly into a single late-onset compartment. Therefore, our observation also indicated the close correlation between the compartments formed by birth date-related PCs and olivocerebellar projections. Furthermore, we found that the cerebellum was composed of three units showing lateral-to-medial developmental gradients, as revealed by the birth dates of PCs. The results suggest that PC birth dates play an important role in organizing cerebellar compartmentalization. J. Comp. Neurol. 519:2594–2614, 2011.

INDEXING TERMS: cerebellum; adenovirus; Purkinje cell; birth date; aldolase C; olivocerebellar projection

The cerebellar cortex is organized into a series of longitudinal compartments along the mediolateral axis (for reviews see Voogd and Glickstein, 1998; Ito, 2001, 2005; Apps and Garwicz, 2005; Apps and Hawkes, 2009). Purkinje cells (PCs) in each compartment are innervated by a specific subarea of the inferior olive (IO) through climbing fibers (olivocerebellar projection) and project their axons to a specific region in the cerebellar or vestibular nuclei (corticonuclear projection). Therefore, the olivocorticonuclear circuit shows modular formation and compartmentalizes the cerebellum. Furthermore, the cerebellar compartments are identified by the expression of a variety of marker genes (for reviews see Hawkes, 1997; Herrup and Kuemerle, 1997; Oberdick et al., 1998; Larouche and Hawkes, 2006; Sillitoe and Joyner, 2007; Apps and Hawkes, 2009). The striped expression of zebrin II (aldolase C), a late-onset marker of cerebellar compartments, is closely correlated with the olivocerebellar and corticonuclear projections (Gravel et al., 1987; Wassef et al., 1992; Voogd et al., 2003; Voogd and Ruigrok, 2004; Sugihara and Shinoda, 2004; Odeh et al., 2005; Sugihara and

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*CORRESPONDENCE TO: Mitsuhiro Hashimoto, Hashimoto Research Unit, RIKEN BSI, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan. E-mail: mhashimoto@brain.riken.jp

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Quy, 2007; Ruigrok et al., 2008) and with physiological activity in the adult cerebellum (Chen et al., 1996; Apps and Garwicz, 2005; Gao et al., 2006; Sugihara et al., 2007; Pijpers et al., 2008). Therefore, it is thought that the cerebellar compartments are established by a specific molecular mechanism and that they are the basic and functional subdivisions of the cerebellum.

The compartments observed in the embryonic cerebellum are distinct from those in the adult cerebellum (for review see Herrup and Kuemerle, 1997). The embryonic compartments are identified by the striped expression of genes that are called "early-onset" markers (e.g., *En-2* and *Wnt7b*; Millen et al., 1995), whereas the adult compartments are identified by other genes, which are called "late-onset" markers (e.g., zebrin II; Leclerc et al., 1988; Tano et al., 1992; Herrup and Kuemerle, 1997). The adult cerebellum is composed of more complex compartments than the embryonic cerebellum (Herrup and Kuemerle, 1997). Moreover, the striped expression of early-onset markers largely disappears after birth (Millen et al., 1995), whereas that of late-onset markers is observed only in the adult cerebellum (Tano et al., 1992). Therefore, the difference between the embryonic and the adult compartments and the transient disappearance of the cerebellar compartments have made it difficult to determine how mediolateral (M-L) compartments are established and how the embryonic compartments (early-onset pattern) and the adult compartments (late-onset pattern) are related, despite several studies having addressed this issue (Larouche and Hawkes, 2006; Marzban et al., 2007; Sillitoe et al., 2009).

By using an adenoviral vector system (Hashimoto and Mikoshiba, 2004), we have found that the pattern of the cerebellar compartments is determined by the selective distribution of Purkinje cells (PCs) that share the same birth date (Hashimoto and Mikoshiba, 2003). In the embryonic cerebellum, the compartments formed by birth date-related PCs (PC birth date compartments) are identical to the compartments defined by the early-onset markers (Fig. 1A; *En2*, *Wnt7B*). Moreover, the relative position of each PC birth date compartment essentially remains stable from embryo (Fig. 1B–G) to adult (Fig. 1H–J). Consequently, our adenoviral vector system allows us to examine the relationship between the embryonic and the adult compartments by comparing the distribution of the birth date-related PCs with the expression of the late-onset marker zebrin II in the adult cerebellum. The results indicate that PC birth dates are critical for the establishment of longitudinal compartments in the mouse adult cerebellum. We speculate that PC birth dates provide essential triggers for the timing of gene expression, cerebellar patterning, and formation of specific projections in the cerebellum.

MATERIALS AND METHODS

Preparation and injection of adenoviral vectors

The AdexCAG-NL-LacZ adenoviral vector (Hashimoto et al., 1996; Hashimoto and Mikoshiba, 2003) expresses nuclear-targeted β-galactosidase (NL-LacZ) under the control of the CAG promoter (Niwa et al., 1991). This vector was based on human adenovirus type 5 (Ad5) but is replication incompetent because it lacks the E1A, E1B, and E3 genomic regions. Each clone was verified by restriction enzyme digestion and PCR for E1A (Zhao et al., 1998) in order to exclude parental adenoviruses (Ad5). Adenoviral vectors were purified and concentrated by double cesium step gradient centrifugation (Kanegae et al., 1994). A high-titer viral stock [1×10^{11} plaque forming units (p.f.u)/ml in 10% (v/v) glycerol/PBS] was stored at −80°C. The titers of the viral stocks were determined by plaque assays on HEK293 cells.

Pregnant ICR mice (Slc:ICR; Nippon SLC, Shizuoka, Japan) were housed in a controlled environment (RIKEN BSI animal facility) under a regulated 12-hour light/dark cycle. The RIKEN BSI Animal Committee approved all procedures involving animal preparation. The day on which the vaginal plug was detected was counted as E0.5.

Mouse embryos were manipulated as described previously (Hashimoto and Mikoshiba, 2004). ICR mice with E10.5, E11.5, and E12.5 pregnancies were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight). AdexCAG-NL-LacZ (total 1×10^8 p.f.u.) was injected into the midbrain ventricle of embryos with a heat-pulled glass micropipette using a microinjector (IM-300; Narisige Japan). After injection, the embryos were carefully replaced into the abdominal cavity, and the body wall muscle was closed with sutures and the skin with surgical staples. At E18.5, the surviving embryos were quickly delivered by caesarian section, and fostered to dams. The next day was defined as postnatal day 0 (P0). Mice were analyzed at 4–8 weeks of age ($n = 3$, E10.5 injection; $n = 5$, E11.5 injection; $n = 5$, E12.5 injection).

Histochemical and immunohistochemical staining

Mice injected with the adenoviral vector were fixed by intracardiac perfusion with PBS, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB; pH 7.4). After dissection, the brains were further fixed in the same fixative overnight at 4°C. To detect the activity of β-galactosidase (β-gal) in the ICR mice injected with AdexCAG-NL-LacZ, the fixed whole brains were stained with 5 mM $K_4[Fe(CN)_6]$, 5 mM $K_3[Fe(CN)_6]$, 1 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, and 0.1%

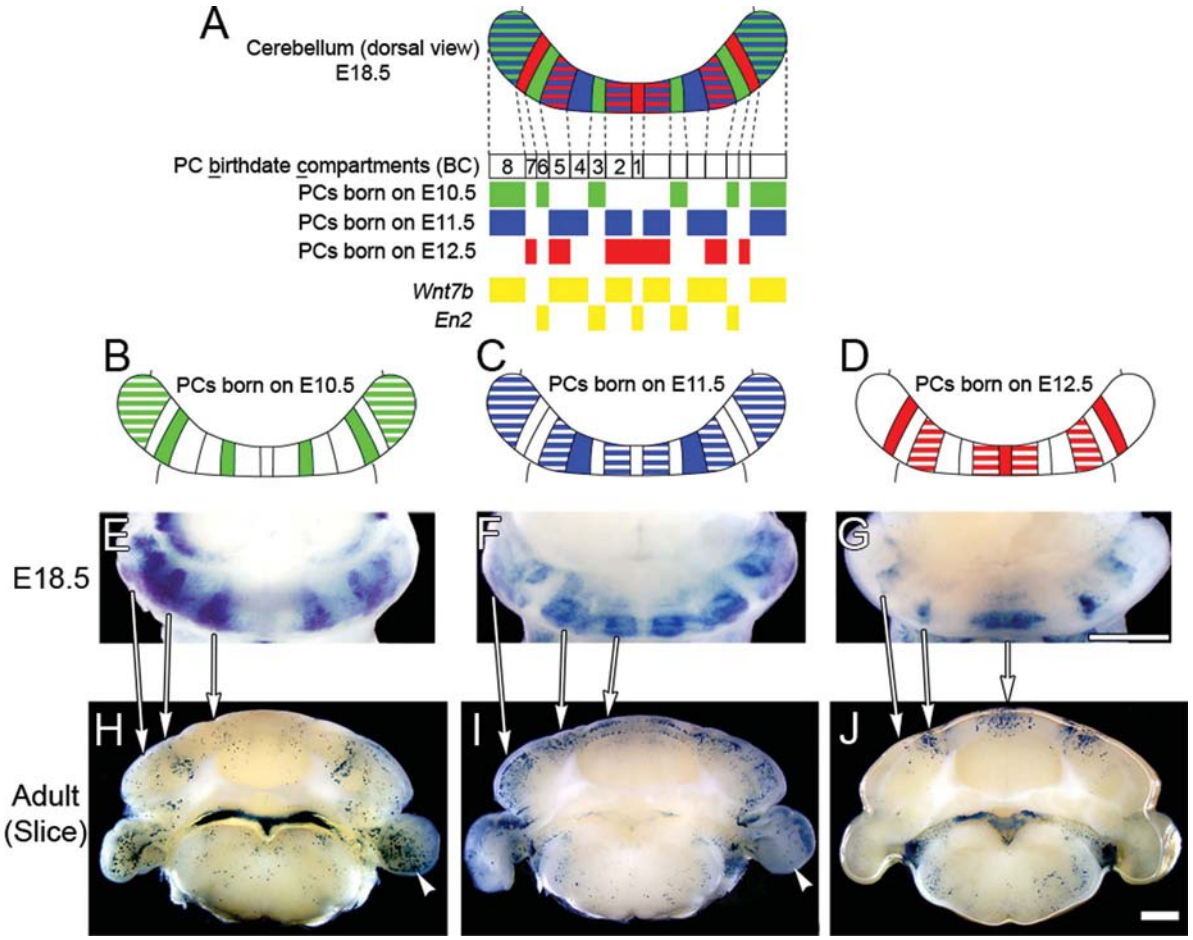


Figure 1. PC birth date compartments in E18.5 and adult cerebella. The distribution of PCs born on E10.5 (green), E11.5 (blue), and E12.5 (red) in E18.5 cerebella are illustrated in **A**. At E18.5, cohorts of PCs born on E10.5, E11.5, and E12.5 form eight longitudinal compartments along the mediolateral axis (designated PC birth date compartments, BC1–BC8). Each cohort of birth date-related PCs is located to a specific subset of compartments displaying nested and, in part, mutual complementarity. PCs born on E10.5 form BC3, BC6, and BC8 (green, **A,B**) PCs born on E11.5 form BC2, BC4, BC5, and BC8 (blue, **A,C**); and PCs born on E12.5 form BC1, BC2, BC5, and BC7 (red, **A,D**). AdexCAG-NL-LacZ was injected into embryos at E10.5, E11.5, and E12.5, and then each cerebellum was whole-mount stained for β-gal (blue color) at E18.5 and at 4 weeks. The dorsal views of E18.5 cerebella (**B–G**) and transverse slices of adult cerebella (**H–J**) are shown caudally. In A, expression patterns of *Wnt7b* and *En2* (Millen et al., 1995; Hashimoto and Mikoshiba, 2003), early-onset markers, are also shown. The arrows indicate the transition from early-onset (E18.5) to late-onset (adult) patterns. PCs born on E10.5 are selectively located to the caudal half of the paraflocculus (PF; arrowhead, H), whereas PCs born on E11.5 are selectively located to the rostral half of the PF (arrowhead, I). PCs born on E12.5 cannot be observed from the dorsal side of PF. Scale bars = 1 mm in G (applies to E–G); 1 mm in J (applies to H–J).

halogenated indoyl-β-D-galactoside (Bluo-gal; Invitrogen, Tokyo, Japan) for 12 hours. After staining, whole brains were cryoprotected in 20% (w/v) sucrose in PBS. The brains were then embedded in 10% (w/v) gelatin in PBS and transversely sectioned (40 μm thick) with a cryomicrotome (SM2000 R; Leica, Solms, Germany) as described previously (Sugihara and Shinoda, 2004; Sugihara and Quy, 2007).

These sections were immunostained with a rabbit polyclonal antibody against zebrin II (see below under Antibody characterization). In brief, the sections were incubated with antizebrin II antibody (320 ng/ml) in PBS with 0.15% (w/v) Triton X-100 (PBST) for 72 hours at 4°C. The sections were washed several times with PBST and then incubated overnight with the goat biotinylated anti-rabbit antibody (1:200; Vector Laboratories, Burlingame, CA). After incubation, the sections were washed several times with PBST and incubated again with the biotinylated peroxidase-avidin complex (Vectastain Standard Elite; Vector Laboratories) in PBST for 6 hours at 4°C. The immunoreactivity of antizebrin II was visualized with 3′-diaminobenzidine tetrahydrochloride (DAB; 1 mg/ml; Dojindo Molecular Technologies, Tokyo, Japan), resulting in a brown precipitate at places where zebrin II was present. After immunostaining, the

sections were sequentially mounted on glass slides (Matsunami Glass, Osaka, Japan). Sections were then counterstained with Nuclear Fast Red (Vector Laboratories) and coverslipped with Permount (Fisher Scientific, Somerville, NJ) according to standard procedures.

Transverse sections of an adult cerebellum were washed several times with PBS and incubated for 1 hour at 4°C in PBS with 1% (w/v) skim milk (Wako, Tokyo, Japan) and 0.1% (w/v) Triton X-100, followed by incubation with the rabbit polyclonal antibody against mouse heat shock protein 25 (Hsp25; 1:250; SPA-801; StressGen, Ann Arbor, MI) diluted with PBST for 2 hours at 4°C. The sections were washed several times with PBST and incubated with the goat biotinylated anti-rabbit antibody (1:200; Vector Laboratories) for 2 hours at 4°C. After incubation, the sections were rinsed several times with PBST and incubated with the biotinylated peroxidase-avidin complex (Vectastain Standard Elite; Vector Laboratories) in PBST for 1 hour at 4°C. The immunoreactivity of anti-Hsp25 was visualized with DAB (1 mg/ml; Dojindo Molecular Technologies).

Antibody characterization

Synthetic peptides of CGAATEEFIKRAEMNGLAAQGKYE that consisted of the amino acid sequence 322–344 of rat aldolase C, which is identical to zebrin II (Ahn et al., 1994), were conjugated to keyhole limpet hemocyanin (KLH). The immunogen was inoculated into rabbits to generate the polyclonal antibody against zebrin II. Specific antibody against zebrin II was affinity purified from the immunized rabbit plasma by affinity columns conjugated to the synthetic peptide of zebrin II. The purified zebrin II antibody recognized a single band of 39 kDa on Western blots of rat cerebellum and stained the cell body and dendrites of PCs in adult rat and mouse cerebella, but no staining was seen in the cerebellar sections after preadsorption with the synthetic peptide of zebrin II (Sugihara and Shinoda, 2004; Sugihara and Quy, 2007).

Rabbit polyclonal anti-Hsp25 antibody was generated against recombinant mouse Hsp25. The anti-Hsp25 antibody recognizes an ~25-kDa protein, which corresponds to the apparent molecular mass of mouse Hsp25, on Western blots of mouse cerebellum and immunostains the cell body and dendrites of PCs in an adult mouse cerebellum. There is no Hsp25 immunostaining in the mouse cerebellar sections after preadsorption with the recombinant mouse Hsp25 (Armstrong et al., 2000).

Analysis of the correlation between PC birth date compartments and zebrin II bands

The images of all stained sections were digitized using an upright microscope equipped with a slide-scanning system (digital virtual microscopy system, VS-100; Olympus, Tokyo, Japan) that scans entire slides at high resolution (0.28 μm/pixel) and high fidelity. All image data were stored on a computer by VS-ASW software (Olympus) and manipulated in Photoshop CS3 (Adobe Systems, San Jose, CA) to adjust white balance of each image. All section images were printed on paper (297 mm × 420 mm) to preserve relative size of all sections, and zebrin II-positive and -negative bands were traced, and then the names of all zebrin II bands and the names of all cerebellar lobules were plotted on each image (e.g., Fig. 3B,C,F–H). Using this anatomical atlas indicating the distribution of PCs born on E10.5, E11.5, and E12.5, which were positive for β-gal, and the zebrin II bands, we examined the correlation between PC birth date compartments and zebrin II bands (e.g., Fig. 3I,J) in the whole cerebellum. The distribution of β-gal-positive PCs and the zebrin II-positive and -negative bands in the whole cerebellum were reconstructed from serial sections of each cerebellum, and then the distribution of β-gal-positive PCs and the zebrin II-positive and -negative bands were plotted on the schematic diagram of the unfolded cerebellar cortex (Fig. 2), as described previously (Sugihara and Shinoda, 2004; Sugihara and Quy, 2007).

Statistical analysis

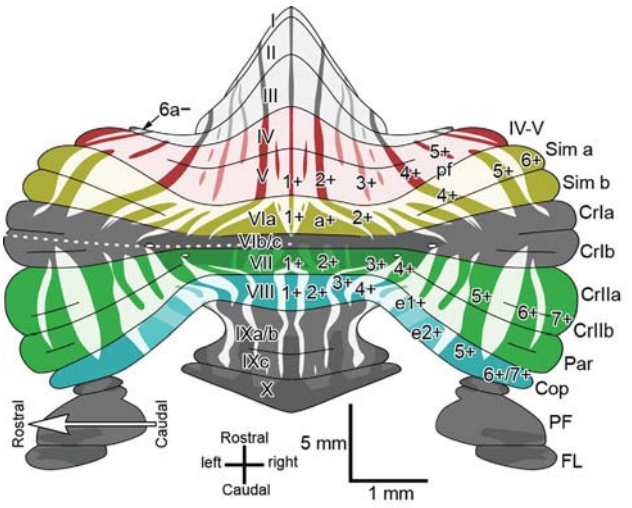
The number of β-gal-positive PCs on each zebrin II band of the transverse region from lobule VII to crus II and paramedian lobule (VII-CrII/Par; Fig. 2, green) was counted (n = 3, E10.5 injection; n = 4, E11.5 injection; n = 4, E12.5 injection). In the present study, VII-CrII/Par consisted of about 50 continuous sections (40 μm thick). The average of β-gal-positive PCs and the standard deviation (SD) of each average were calculated. We used the Tukey-Kramer method to calculate statistical significance (JMP; SAS Institute Japan, Tokyo, Japan). Statistical significance was assumed at $P < 0.01$.

RESULTS

In the present study, we designated PC birth date compartments as BC1 (the most medial) to BC8 (the most lateral), according to the pattern seen in the E18.5 cerebellum (Fig. 1), in order to distinguish them from zebrin II bands (Fig. 2). All zebrin II bands were named according to a previous report (Sugihara and Quy, 2007). Positive and negative signs (e.g., 4+ and 4a–) indicate zebrin II-immunopositive and -negative bands, respectively (Fig. 2).

Double labeling with an adenoviral vector and an antizebrin II antibody

Our adenoviral vector system allows us to introduce transgenes into neuronal progenitor cells in a neuronal



	Transversal regions		Name of zebrin II-bands									
	Vermis	Paravermis / Hemisphere										
1	IV-V		1+/−	2+/−	b+/−	3+/−	4+/−	5+/−	6+	6a−		
2	VIa	Sim	1+/−	a+/−	2+/−	2b+/−	c+/−	d+/−	4+/−	5+/−	6+	
3	VII	CrII/Par	1+/−	2+/−	3+/−	4+	4a−	4b+/−	5a+/−	5+/−	6+/−	7+
4	VIII-Cop		1+/−	2+/−	3+/−	4+/−	f+/−	e1+/−	e2+/−	5+/−	6+	7+

Figure 2. An unfolded representation of zebrin II bands in the entire mouse cerebellum. This drawing is adapted from Sugihara and Quy (2007). The patterns of the zebrin II bands clearly differ in the four transverse regions: lobule IV and V (IV-V; red), lobule VIa to simple lobule a/b (VIa-Sim; yellow), lobule VII to Crus II a/b and paramedian lobule (VII-CrII/Par; green), and lobule VIII to copula pyramidis (VIII-Cop; blue). The names of the zebrin II bands on the right side of the four transverse regions are listed at bottom. In the transverse region from lobule VIb/c to Crus Ib (white dotted line), zebrin II-positive bands appear to merge together, so there are no zebrin II-negative bands. The rostrocaudal direction in FL and PF regions is equivalent to the lateromedial direction in other parts of the cerebellar cortex (arrow). Cop, copula pyramidis; Crla, Crus Ia; Crlb, Crus Ib; CrIIa, Crus IIa; CrIIb, Crus IIb; Par, paramedian lobule; pf, primary fissure; Roman numerals, lobule number; Sim a, simple lobule a; Sim b, simple lobule b.

birth date-specific manner (Hashimoto and Mikoshiba, 2004). This technique is very useful for the examination of neuronal development and function because we can genetically manipulate each subset of neurons sharing the same neuronal birth date and can examine the native behavior of each such neuronal subset. PCs are generated from progenitor cells located on the roof of the fourth ventricle between E10.5 and E12.5. PCs born on E10.5 (Fig. 1E,H), E11.5 (Fig. 1F,I), and E12.5 (Fig. 1G,J) are labeled with nuclear-targeted β-galactosidase by the injection of the adenoviral vector AdexCAG-NL-LacZ into the midbrain ventricle of mouse embryos at E10.5, E11.5, and E12.5, respectively. We find that PCs that are generated at E10.5, E11.5, and E12.5 form distinct subsets of compartments, which are arranged mediolaterally (BC1–BC8) in the E18.5 cerebellum (Fig. 1). PCs born on E10.5 form BC3, BC6, and BC8 (Fig. 1A,B, green); PCs born on E11.5 form BC2, BC4, BC5, and BC8 (Fig. 1A,C, blue); and PCs born on E12.5 form BC1, BC2, BC5, and BC7 (Fig. 1A,D, red). Therefore, the pattern of mediolateral compartments in the cerebellum is determined by the birth date of PCs. The patterns of PC birth date compartments in the embryonic cerebellum are similar to the expression patterns of several early-onset markers (Fig. 1A, *En2*, *Wnt7b*; Hashimoto and Mikoshiba, 2003). However, in contrast to these early, and also late-onset markers such as zebrin II, the adenoviral labeling of PCs is stable from embryo until adult (Fig. 1E–J; at least until 1.5 years of age; data not shown). Therefore, by comparing PC birth date compartments and zebrin II bands in adult cerebella, we were able to examine the correlation and the transition between early- and late-onset cerebellar compartmentalization.

The adenoviral vector AdexCAG-NL-LacZ was injected into E10.5, E11.5, and E12.5 embryos, and the brains [n = 3, E10.5 injection (Fig. 1H); n = 5, E11.5 injection (Fig. 1I); n = 5, E12.5 injection (Fig. 1J)] were fixed when they were 4–8 weeks old. The cerebella were whole-mount stained for β-gal. PC birth date compartments were reproducible, and the pattern of PC birth date compartments was stable in each adult cerebellum (data not shown). A series of transverse sections from the β-gal stained brain

Figure 3. Double labeling with an adenoviral vector and antizebrin II antibody. AdexCAG-NL-LacZ was injected into embryos at E11.5 (**A**) and E12.5 (**E**), and then each cerebellum was whole-mount stained for β-gal (blue color) at P27. Each stained cerebellum was transversely sectioned, then stained with antizebrin II antibody (brown color) and counterstained with nuclear fast red (red color). **B–D** and **F–H** indicate the double-labeled sections taken from A and B, respectively. The letters and numerals (e.g., a+/−, 5+/−) indicate the identities of zebrin II bands. **I** and **J** show magnifications of the double-labeled sections, and the bars at bottom in I and J indicate the zebrin II-positive (brown color) and β-gal-positive (blue color) regions in each section. Zebrin II immunoreactivity is observed in the dendrite (I, arrow) and cytosol (I, black arrowhead) of PCs. In contrast, β-gal staining is observed in the cell bodies of PCs in each PC birth date compartment (I, white arrowhead). Some of the PCs in the PC birth date compartment are faintly labeled for β-gal (J, open arrow). DPF, dorsal paraflocculus; g, granule cell layer; m, molecular layer; p, Purkinje cell layer; VPF, ventral paraflocculus. Scale bars = 1 mm in A–H; 0.1 mm in I,J.

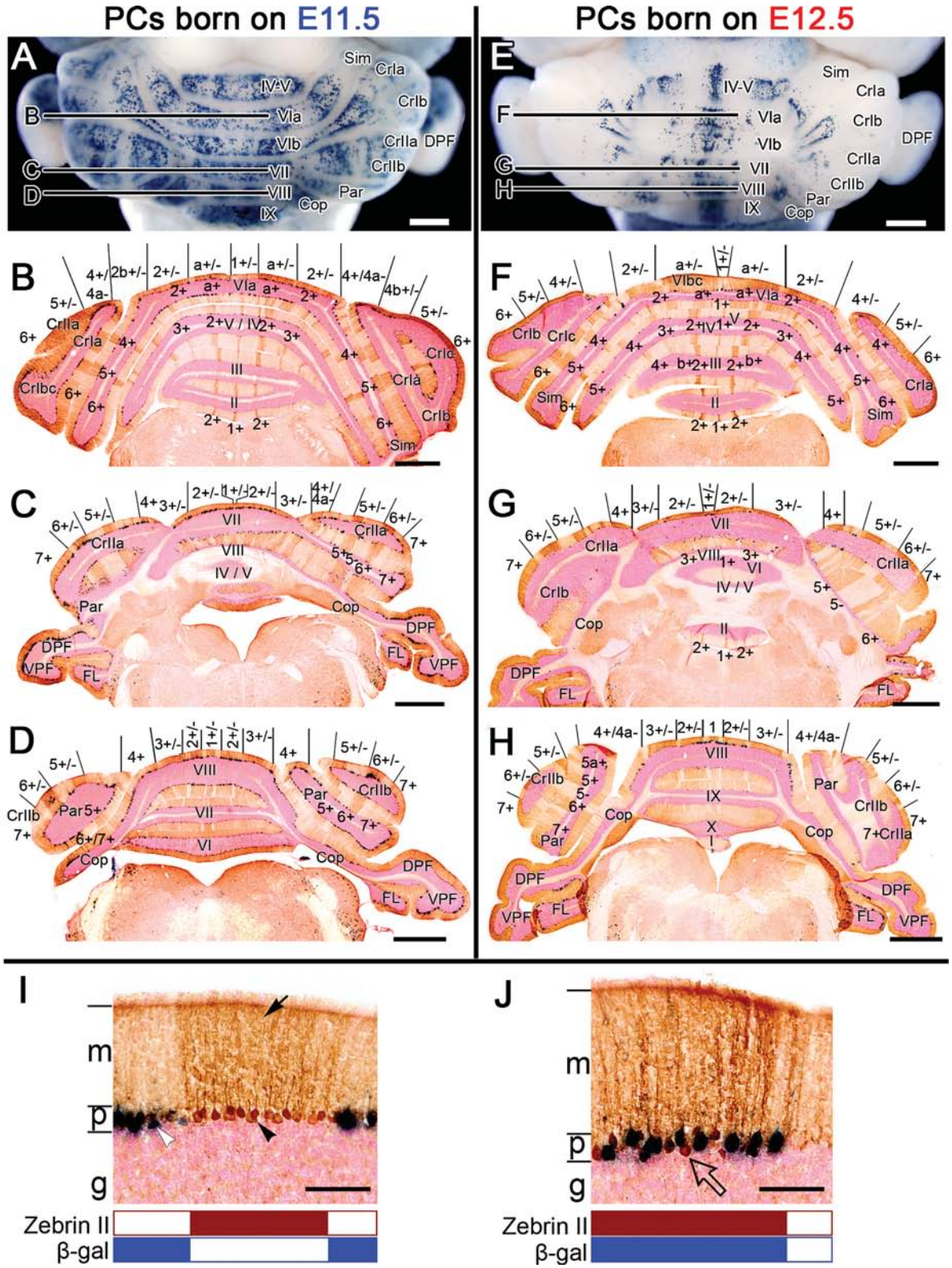

PCs born on E11.5
PCs born on E12.5
A
B
C
D
E
F
G
H
I
J
Sim
Crla
Crlb
Crlla
DPF
Crllb
Par
Cop
IV-V
VIa
VIb
VII
VIII
IX
m
p
g
Zebrin II
β-gal

Figure 3

was immunostained with antizebrin II antibody (Fig. 3B–D,F–J). Zebrin II immunoreactivity was observed in the dendrites (Fig. 3I, arrow) and cytosol of PCs (Fig. 3I, black arrowhead) and revealed zebrin II-positive and -negative bands in the cerebellum (Fig. 3I,J). In contrast, β-gal staining was observed in cell bodies of PCs (Fig. 3I, white arrowhead). The β-gal-positive PCs formed compartments in the cerebellum (Fig. 3I,J). Therefore, it was easy to distinguish zebrin II bands from β-gal-positive PC birth date compartments. Adenoviral injection and β-gal staining never altered the multiple and striped pattern of zebrin II immunostaining compared with controls (data not shown). PC birth date compartments, which were composed of β-gal-positive PCs, coincided with zebrin II-negative (Fig. 3I) and -positive (Fig. 3J) bands. Some of PCs in a PC birth date compartment seemed to be faintly labeled with β-gal (Fig. 3J, open arrow). This seems to be caused by the difference in efficiency of the adenoviral infection. When AdexCAG-NL-LacZ is injected into the ventricle of embryonic brain, the adenoviral particles randomly infect PC progenitor cells on the surface of the fourth ventricle (Hashimoto and Mikoshiba, 2003, 2004; Hashimoto and Hisano, 2011). Therefore, the multiplicity of infection (moi) of AdexCAG-NL-LacZ is different on every other PC progenitor cell. Probably, the moi of AdexCAG-NL-LacZ on PCs that are faintly labeled with β-gal is relatively low.

Distribution of birth date-related PCs in the entire cerebellar cortex

A previous study (Sugihara and Quy, 2007) has defined zebrin II bands throughout the mouse cerebellar cortex and provides a detailed map of zebrin II bands in the adult cerebellum (Fig. 2). Using this map, we plotted the names and the locations of zebrin II-positive and -negative bands on all pictures of transverse sections of the cerebella (e.g., Fig. 3B,C,F–H). To clarify the distribution of PCs born on E10.5, E11.5, and E12.5 in adult cerebella, we systematically compared the location of the β-gal-positive PCs with zebrin II-positive and -negative bands on each transverse section. The distribution of β-gal-positive PCs and the zebrin II-positive and -negative bands in the whole cerebellum were reconstructed from the serial sections of each cerebellum. According to the previous study (Sugihara and Quy, 2007), we plotted the reconstructed distribution of β-gal-positive PCs and the reconstructed zebrin II bands on the schematic diagram of the unfolded cerebellar cortex (Fig. 4A–C). The schematic diagrams indicated the correlation between PC birth date compartments and zebrin II bands on the entire cerebellar cortex (Fig. 4A–C). We found that PCs born on E10.5 (Fig. 4A), E11.5 (Fig. 4B), and E12.5 (Fig. 4C) were selectively located in the striped regions in the adult cerebella and formed the longitudinal compartments in the adult cerebellum. The distributions of the birth date-related PCs display bilateral symmetry and are almost complementary to each other. They were consistent with PC birth date compartments in E18.5 cerebella (Fig. 1B–G). In addition, a similar distribution of labeled PCs is observed in the rostral (including IV-V and VIa-Sim) and caudal (including VII-CrII/Par and VIII-Cop) cerebellar cortices (Fig. 4). Furthermore, these compartments formed by PCs born on E10.5, E11.5, and E12.5 closely matched zebrin II bands in the entire cerebellum. These observations indicated that PC birth date compartments in the adult cerebellum correlated strikingly with zebrin II bands.

PC birth date compartments and zebrin II bands are closely correlated in the adult cerebellum

Zebrin II bands are useful to examine the cerebellar compartmentalization because the pattern of zebrin II bands (Fig. 2) is stable between individual mice. However, zebrin II bands are complex (Fig. 2). For instance, the names of zebrin II bands in the rostral and caudal halves of the cerebellum differ clearly from one another. In addition, zebrin II-positive bands appear to merge together in the transverse region from lobule VIb/c to CrIb (VIb/c-CrIb; Fig. 2, white dotted line); thus, there are no names of zebrin II bands in VIb/c-CrIb. Therefore, the complicated nomenclature of zebrin II bands has introduced confusion into the study of cerebellar mediolateral compartmentalization (Apps and Hawkes, 2009) and has made examination of the correlation between PC birth date compartments and zebrin II bands difficult. Therefore, we divided the cerebellum into multiple transverse regions: 1) lobule IV and V (IV-V; Fig. 2, red), 2) from lobule VIa to simple lobule sublobule a and b (VIa-Sim; Fig. 2, yellow), 3) from lobule VII to Crus IIa and IIb and paramedian lobule (VII-CrII/Par; Fig. 2, green), and 4) from lobule VIII to copula pyramidis (VIII-Cop; Fig. 2, light blue). The IV-V region is located in the anterior lobe, which has no structural boundary between the vermis and the hemispheres, and consists of four major (1+, 2+, 4+, and 6+) and several narrow (b+, 3+, and 5+) zebrin II-positive bands. Additionally, there is a narrow satellite zebrin II-negative band (6a–) in the medial part of 6+. The VIa-Sim region is located in the posterior lobe, in which the paravermal sulcus separates the vermis (VIa) and hemisphere (simple lobule). The VIa-Sim contains nine zebrin II-positive bands (1+, a+, 2+, 2b+, c+, d+, 4+, 5+, and 6+). Zebrin II bands a+, 2b+, c+, and d+ are present only in this region. The VII-CrII/Par region is located in the posterior lobe, in which the vermis (VII) and

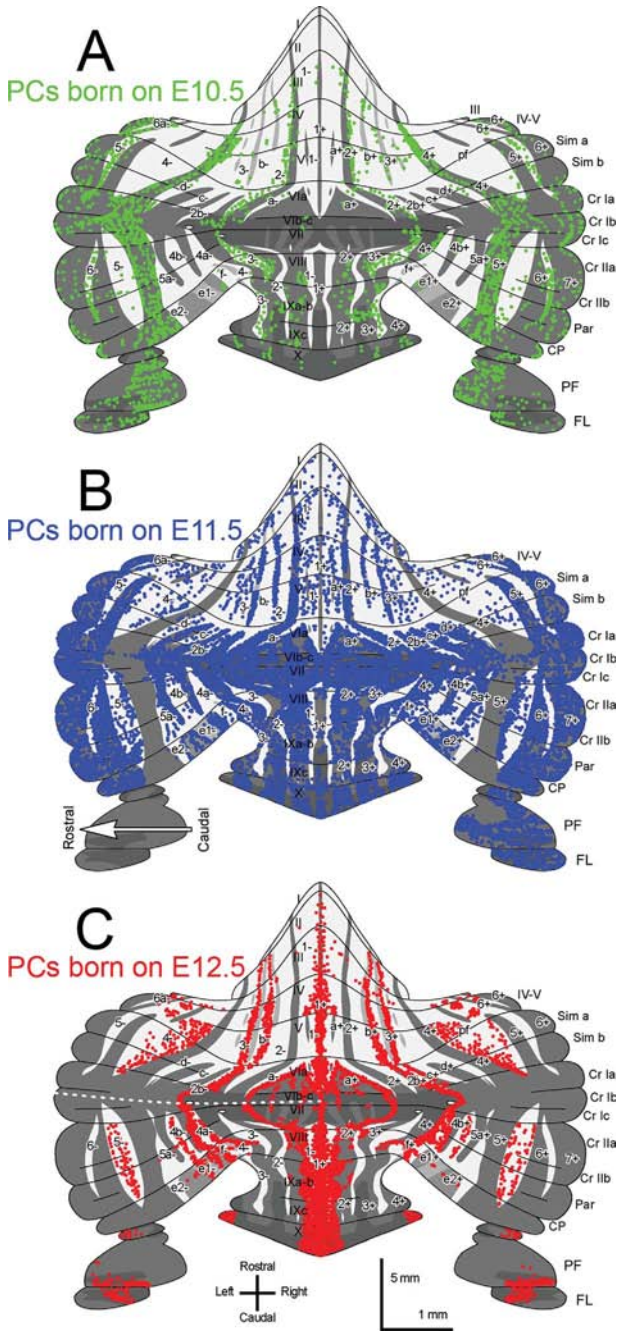


Figure 4. Distribution of PCs born on E10.5, E11.5 and E12.5 in the adult cerebellum. Distributions of PCs born on E10.5 (**A**, green dots), PCs born on E11.5 (**B**, blue dots), and PCs born on E12.5 (**C**, red dots), which are positive for β-gal, are plotted on the topographic map of zebrin II bands (Fig. 2) for the entire adult cerebellum. The left FL and PF of B were lost.

hemisphere (crus IIa, crus IIb and paramedian lobule) are clearly separated by the paravermal sulcus. The VII-CrII/Par contains nine zebrin II-positive bands (1+, 2+, 3+, 4+, 4b+, 5a+, 5+, 6+, and 7+). The VIII-Cop region is located in the caudal edge of the cerebellum and has a shallow paravermal sulcus between the vermis and the hemisphere, which contains six major zebrin II-positive bands (1+, 2+, 3+, 5+, 6+, and 7+) and four weakly zebrin II-immunostained bands (f+, e1+, e1–, and e2+). Zebrin II band 6–, which would be intercalated between 6+ and 7+, is absent in this region.

To clarify the detailed correlation between PC birth date compartments in the adult cerebellum and zebrin II bands, we systematically compared the distribution of β-gal-positive PCs with all zebrin II bands in all sections belonging to IV-V (Fig. 2, light red; Fig. 6), VIa-Sim (Fig. 2, light yellow; Fig. 7), VII-CrII/Par (Fig. 2, light green; Fig. 8), and VIII-Cop (Fig. 2, light blue; Fig. 9). In the case of compartments formed by PCs born on E10.5, a few labeled PCs were observed in a zebrin II band in given sections (Figs. 6A–C, 7A,B, 8A–C, 9A,B). However, the β-gal-positive PCs were also observed in the same zebrin II band in adjacent serial sections. Furthermore, PCs born on E10.5 were localized to the same zebrin II bands and formed the longitudinal compartments in the adult cerebellum (Figs. 1H, 4A). We counted the number of PCs born on E10.5, E11.5, and E12.5 in each zebrin II band of VII-CrII/Par ($n = 3$, E10.5 injection; $n = 4$, E11.5 injection; $n = 4$, E12.5 injection; Fig. 5). PCs born on E10.5 (Fig. 5, green) were selectively located in 3–, 5+, and 6– zebrin II bands. PCs born on E11.5 (Fig. 5, blue) solely formed zebrin II bands 3+, 4+, 5a+/–, 6+, and 7+, and PCs born on E12.5 (Fig. 5, red) solely formed zebrin II bands 1+/– and 4a–. The difference in the total cell number between zebrin II bands is probably caused by the difference in the shape (width and length) of the bands (refer to Fig. 2). Therefore, even if a few β-gal-positive PCs were observed in a zebrin II band in a section, we considered that there was a correlation between the compartments formed by PCs born on E10.5 and the zebrin II band.

Although the pattern of zebrin II bands was different and the names of zebrin II bands are not consistent between IV-V (Fig. 6), VIa-Sim (Fig. 7), VII-CII/Par (Fig. 8), VIII-Cop (Fig. 9), and lobule IX (Table 1), the observations clearly indicated that PC birth date compartments and zebrin II bands were closely correlated and how individual PC birth date compartments in the embryonic cerebellum were transformed into individual zebrin II bands in the adult cerebellum. Consequently, we found that the transitions from PC birth date compartments in the embryonic cerebellum (early-onset pattern) to zebrin II bands in the adult cerebellum (late-onset pattern) were categorized into two types of groups: 1) a single PC birth date compartment directly became a single zebrin II-positive or -negative band in the adult cerebellum and 2) a single PC birth date compartment was transformed into multiple zebrin II-positive and -negative bands in the adult cerebellum. In addition, PC birth date compartments belonging to the type 1 group were formed by a single birth cohort

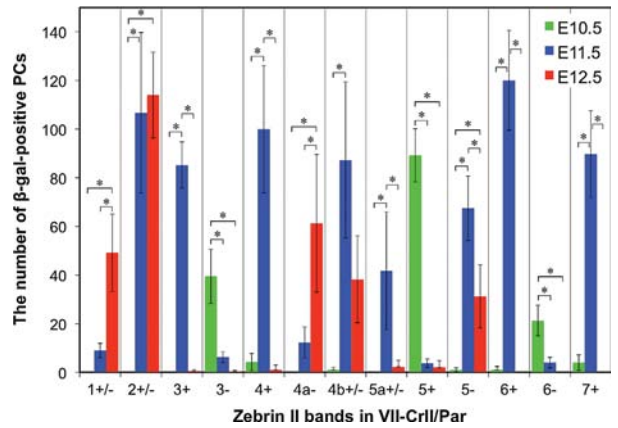


Figure 5. Number of β-gal-positive PCs in each zebrin band of VII-CrII/Par. Numbers of PCs born on E10.5 (green bars), E11.5 (blue bars), and E12.5 (red bars), which were labeled with β-gal, are counted in each zebrin II band of VII-CrII/Par (n = 3, E10.5 injection; n = 4, E11.5 injection; n = 4, E12.5 injection). The average of β-gal-positive PCs born on E10.5, E11.5, and E12.5 is indicated in each zebrin II band. PCs born on E10.5 are selectively located in 3–, 5+, and 6– zebrin II bands. PCs born on E11.5 solely form zebrin II bands 3+, 4+, 5a+/–, 6+, and 7+. PCs born on E12.5 solely form zebrin II bands 1+/– and 4a–. In contrast, zebrin II bands 2+/–, 4b+/ –, and 5– are composed of PCs born on E11.5 and E12.5. The difference in the total cell number between zebrin II bands seems to be caused by the difference in the shape (width and length) of zebrin II bands (refer to Fig. 2). Bars represent mean ± SD. *$P < 0.01$.

of PCs (E10.5, E11.5, or E12.5). In contrast, PC birth date compartments belonging to the type 2 group were formed by two different birth cohorts of PCs (E11.5 and E12.5). The two different birth cohorts of PCs were intermingle with each other, and, finally, the distribution of the two different birth cohorts of PCs demarcated the positions of zebrin II-positive and -negative bands in the adult cerebellum. The transition from the embryonic compartments (early-onset pattern) to the adult compartments (late-onset pattern) had remarkable similarity in all transverse regions (Figs. 6N, 7M, 8P, 9M).

BC3, BC4, BC6, and BC7 belong to the type 1 group. These PC birth date compartments directly became single zebrin II bands in the adult cerebellum (Table 1, BC3, BC4, BC6, BC7). BC3 and BC6 were formed by PCs born on E10.5 and BC3 became zebrin II-negative bands, whereas BC6 became zebrin II-positive bands (Table 1, BC3, BC6). BC4 was composed of PCs born on E11.5 and became zebrin II-positive bands (Table 1, BC4). BC7 was formed by PCs born on E12.5 and agreed with zebrin II-negative bands (Table 1, BC7). On the other hand, BC2 and BC5 belong to the type 2 group. BC2 and BC5 were formed by PCs born on E11.5 and E12.5 in the embryonic cerebellum (Fig. 1A) and became multiple zebrin II-positive and -negative bands in the adult cerebellum (Table 1, BC2, BC5). PCs born on E11.5 were distributed through BC2 and BC5, whereas PCs born on E12.5 were selectively located in the most medial zebrin II bands in BC2 (Figs. 7M, 8P, 9M; Table 1, BC2) and were preferentially located in the medial region of BC5 (Figs. 7M, 8P, 9M; Table 1, BC5).

BC1 became zebrin II bands 1+ and 1– in the adult cerebellum and was very narrow in most cases. If zebrin II bands 1+ and 1– are assumed to be a single compartment, BC1 also belongs to the type 1 group because BC1 is composed of a single birth cohort of PCs, which are born on E12.5 (Table 1, BC1). In the adult cerebellum, BC8 was clearly divided into three compartments that were designated as BC8a, BC8b, and BC8c (Figs. 7M, 8P; Table 1, BC8). BC8a and BC8c were formed by PCs born on E11.5 and were identical to zebrin II-positive bands (Fig. 8I; Table 1, BC8a, BC8c), whereas BC8b was formed by PCs born on E10.5 and was identical to zebrin II-negative bands (Fig. 8C; Table 1, BC8b). In the embryonic cerebellum, BC8 was formed by PCs born on E10.5 and E11.5 (Fig. 1A–C,E,F); however, PCs born on E10.5 and PCs born on E11.5 were never intermingled with each other in BC8 and formed separate compartments, which were BC8a (E11.5), BC8b (E10.5), and BC8c (E11.5), in the adult cerebellum (Table 1, BC8). Consequently, we think that BC8a, BC8b, and BC8c also belong to the type 1 group, because a single birth cohort of PCs directly became a single zebrin II-positive or -negative band. Overall, in PC birth date compartments belonging to the type 1 group, PCs born on E10.5 directly become zebrin II-positive band (BC6) and zebrin II-negative bands (BC3 and BC8b); however, PCs born on E11.5 exhibit a tendency to become zebrin II-positive bands (BC4, BC8a, and BC8c), and PCs born on E12.5 preferentially become zebrin II-negative band (BC7). Remarkably, the compartments formed by PCs born on E12.5 were identical to zebrin II-negative bands in IV-V (Fig. 6N).

We observed some exceptional cases in this grouping of PC birth date compartments. In IV-V, BC7 could not been distinguished from BC8, because the boundary between BC7 and BC8 was not apparent. Therefore, we cannot categorize BC7 and BC8 in IV-V into the type 1 or type 2 groups. However, the most lateral compartment in IV-V, which may be composed of BC7 and BC8, became multiple zebrin II-positive and -negative bands (4–, 5+, 5–, 6+, 6a–, and 6–) in the adult cerebellum (Fig. 6N). In VIII-Cop, the boundary between BC2 and BC3 was observed on the inside of zebrin II-positive band 3+ (Fig. 9M). Therefore, in this region, the formation of PC birth date compartments is inconsistent with the patterning of zebrin II-positive and -negative bands.

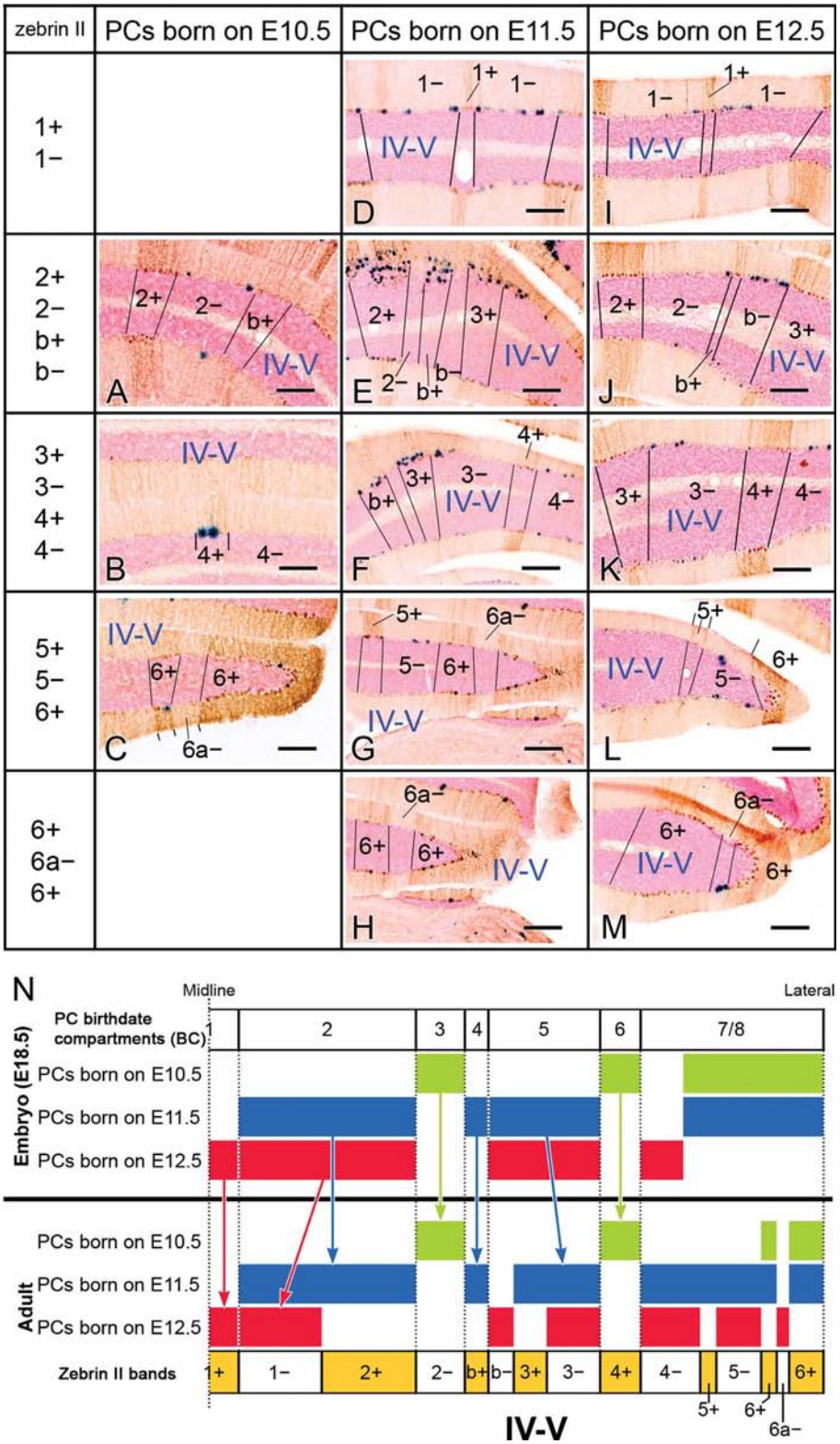


Figure 6. Correlation between PC birth date compartments in the adult cerebellum and zebrin II bands in IV-V. Adult cerebella injected with AdexCAG-NL-LacZ at E10.5 (**A–C**), E11.5 (**D–H**), and E12.5 (**I–M**) were sectioned transversely and double stained for β-gal (blue) and antizebrin II antibody (brown) and counterstained with Nuclear Fast Red (red). β-Gal staining, zebrin II immunoreactivity, and Nuclear Fast Red staining were observed in the PC and molecular and granule cell layers, respectively. The positions and names of zebrin II bands are indicated in A–M. The diagram in **N** indicates a close correlation between the early-onset pattern represented by PC birth date compartments in the E18.5 cerebellum (upper half) and the late-onset pattern represented by zebrin II immunoreactivity in the adult cerebellum (lower half). The arrows in N show the transition from the early-onset pattern to the late-onset pattern. The dorsolateral direction is upward in B,C,F–H,K–M. Scale bars = 250 μm.

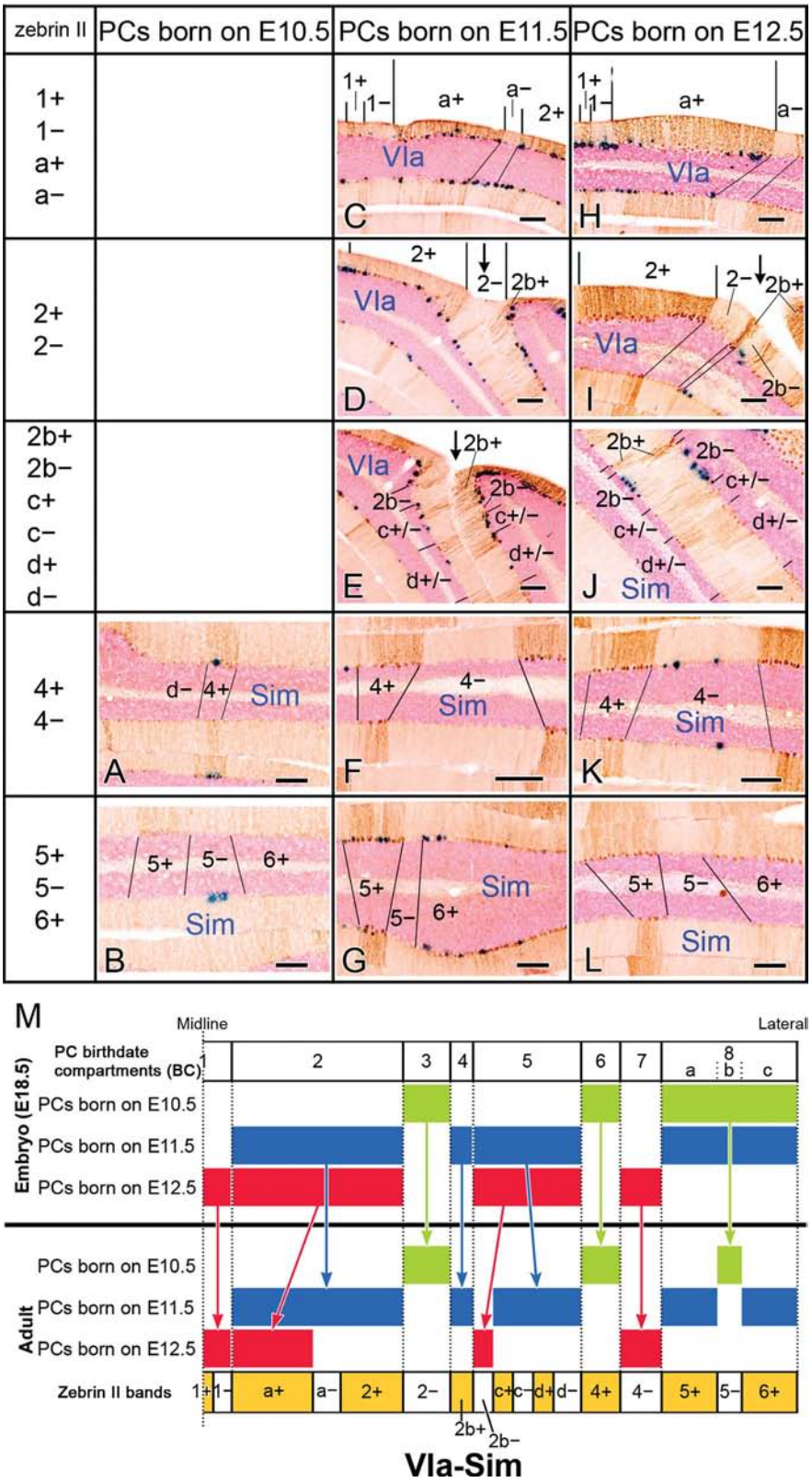


Figure 7. Correlation between PC birth date compartments in the adult cerebellum and zebrin II bands in VIa-Sim. **A–L** are arranged in a manner similar to the panels in Figure 6. The approximate boundary between the vermis and hemisphere is indicated by arrows in D,E,I. The diagram in **M** indicates a close correlation between the early-onset pattern represented by PC birth date compartments in the E18.5 cerebellum (upper half) and the late-onset pattern represented by zebrin II immunoreactivity in the adult cerebellum (lower half). The arrows in M show the change from the early-onset pattern to the late-onset pattern. Scale bars = 250 μm.

PC birth date compartments in paraflocculus and flocculus

Zebrin II expression in the paraflocculus (PF) and the flocculus (FL) did not reveal cerebellar compartments, because all PCs in PF and FL were immunopositive for antizebrin II antibody (Fig. 10A–C). In contrast, the birth date-related PCs compartmentalized PF and FL into several regions. PCs born on E10.5 were selectively located in the caudal half of PF (Fig. 1H, arrowhead; Fig. 4A, PF), whereas PCs born on E11.5 were selectively located in the rostral half of PF (Fig. 1I, arrowhead; Fig. 3A, DPL; Fig. 4B, PF). In contrast, PCs born on E12.5 were situated in the ventromedial region of PF (Fig. 4C, PF; Fig. 10C, arrowhead), which could not be seen from the dorsal side of PF (Fig. 1J, arrowhead; Fig. 3E, DFP). In FL, PCs born on E10.5 were preferentially located in the caudal region (Fig. 4A, FL), and PCs born on E11.5 seemed to be scattered within FL (Fig. 3D, FL; Fig. 4B, FL), whereas PCs born on E12.5 were preferentially situated in the middle of FL (Fig. 3H, FL; Fig. 4C, FL). Consequently, PCs born on E10.5, E11.5, and E12.5 form compartments in PF and FL even though there are no zebrin II-negative bands in PF and FL; furthermore, the compartments formed by PCs born on E10.5, E11.5, and E12.5 seem to be mutually complementary in PF and FL (Fig. 10A–C). In contrast to zebrin II bands, it is known that the immunoreactivity of antiheat shock protein 25 (Hsp25) reveals longitudinal compartments in PF and FL (Armstrong et al., 2000; Sillitoe and Joyner, 2007; Sillitoe et al., 2008; Apps and Hawkes, 2009). To compare PC birth date compartments (Fig. 10A–C) with the Hsp25-immunopositive compartment in PF and FL, we immunostained a section of PC and FL with anti-Hsp25 antibody (Fig. 10D). Hsp25-positive PCs were located in the ventromedial region of PF and the dorsomedial region of FL (Fig. 10D, white arrows). The Hsp25-immunopositive compartment seems to agree with the compartment formed by PCs born on E12.5 in PF and FL (Fig. 10C).

Olivocerebellar projection patterns in relation to PC birth date compartments

The patterns of olivocerebellar and corticonuclear projections divide the cerebellar cortex into multiple longitudinal compartments (Buisseret-Delmas and Angaut, 1993). The olivocerebellar and corticonuclear projection patterns were identified in each zebrin II band throughout the cerebellum, and the close correlation between the longitudinal compartments formed by the olivocerebellar/corticonuclear projections and zebrin II bands has been demonstrated (Gravel et al., 1987; Wassef et al., 1992; Voogd et al., 2003; Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004; Sugihara and Quy, 2007; Sugihara et al., 2009). Because our data indicated a close correlation between PC birth date compartments and zebrin II bands, we examined the correlation between PC birth date compartments and olivocerebellar and corticonuclear projections by comparing PC birth date compartments with zebrin II bands.

PC birth date compartments that were formed by single birth date cohorts of PCs, BC1 (E12.5), BC3 (E10.5), BC4 (E11.5), BC6 (E10.5), and BC7 (E12.5), each developed into a single zebrin II compartment or two neighboring zebrin II compartments. In the other PC birth date compartments that were formed by two different birth date cohorts of PCs, BC2 (E11.5 and E12.5), BC5 (E11.5 and E12.5), and BC8 (E10.5 and E11.5), the two cohorts tended to become segregated into mediolaterally distinct subcompartments that contributed to multiple zebrin II bands in the adult cerebellum. Furthermore, the longitudinal formation of PC birth date compartments in the adult cerebellum (Fig. 4) revealed the linkage between zebrin II bands in the rostral (lobule I-V and VIa-Sim) and caudal (VII-CrII/Par and VIII-Cop) halves of the cerebellum (Table 1). BC1 linked 1+ and 1– throughout all regions in the adult cerebellum. In BC2, a compartment formed by PCs born on E12.5 was located to the medial part of BC2 and linked a+ in VIa-Sim to 2+ in VII-CrII/Par and VIII-Cop. BC3 linked 2– in IV-V and VIa-Sim (rostral cerebellum) to 3– in VII-CrII/Par and VIII-Cop (caudal cerebellum). BC4 linked b+ in IV-V, 2b+ in VIa-Sim, and 4+ in VII-CrII/Par and VIII-Cop. BC6 linked 4+ in the rostral cerebellum to 5+ in the caudal cerebellum. BC7 linked 4– in the rostral cerebellum to 5– in the caudal cerebellum. BC8 in the embryonic cerebellum was reorganized into three parts in the adult cerebellum. The medial (BC8a), intermediate (BC8b), and lateral (BC8d) parts corresponded to 5+, 5–, and 6+ in VIa-Sim and 6+, 6–, and 7+ in VII-CrII/Par and VIII-Cop, respectively, indicating that 5+, 5–, and 6+ in the rostral cerebellum were linked, respectively, with 6+, 6–, and 7+ in the caudal cerebellum.

The linkage between zebrin II bands in the rostral cerebellum and zebrin II bands in the caudal cerebellum, which was revealed by the longitudinal formation of PC birth date compartments, agreed with the topographic pattern of olivocerebellar climbing fiber projections (Table 1). BC1, BC3, BC4, BC6, BC7, BC8a, BC8b, and BC8c were identical to the longitudinal compartments formed by olivocerebellar projections, which were named, respectively, A, X, X-CX, C2, C3, D1, D0, and D2 olivocerebellar zone (Table 1; Voogd et al., 2003; Voogd and Ruigrok, 2004; Sugihara and Quy, 2007). According to the cross-correlation among PC birth date compartments, zebrin II bands, and the topographic map of olivocerebellar projections, we made a list (Table 1) indicating the

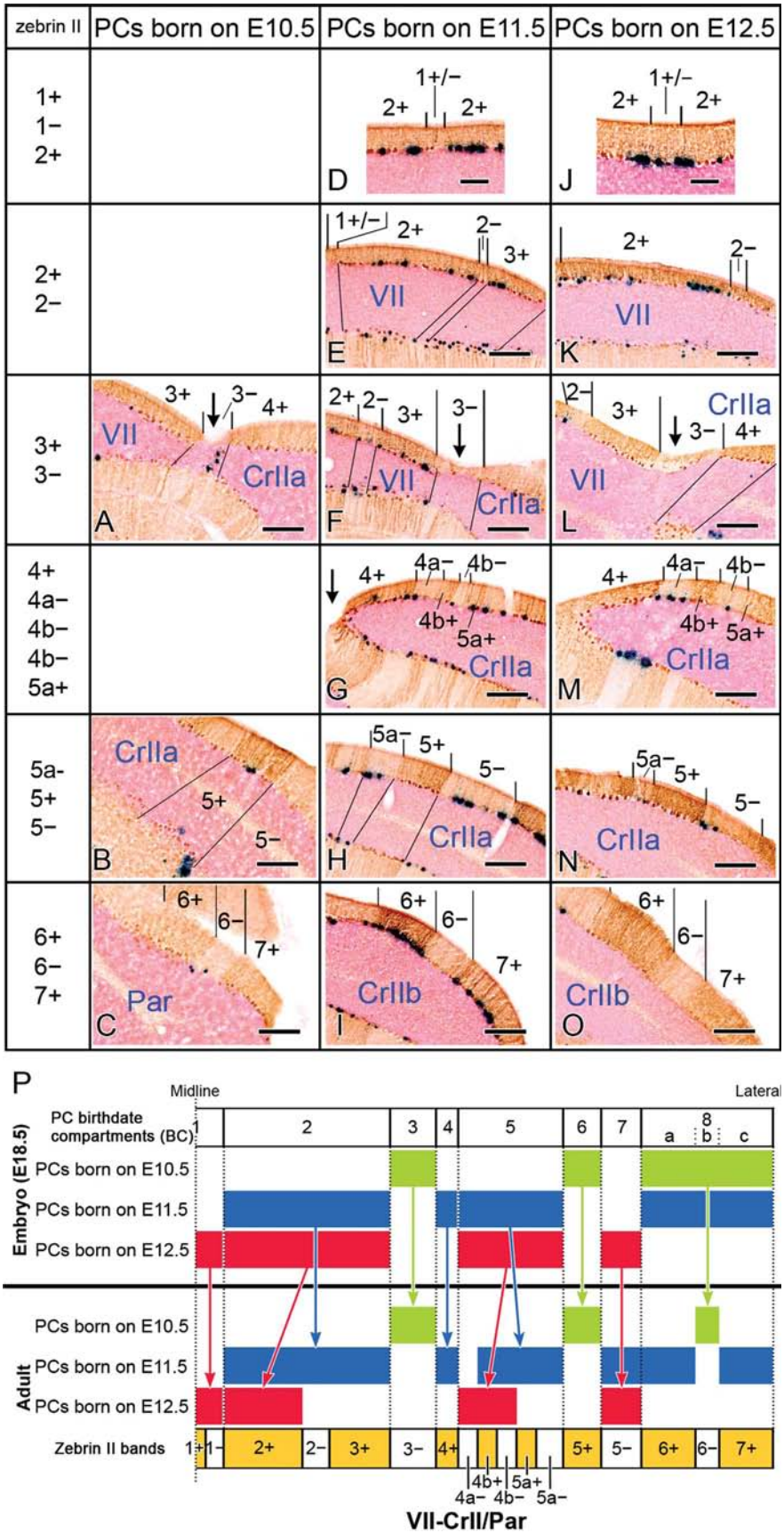

zebrin II
PCs born on E10.5
PCs born on E11.5
PCs born on E12.5
1+
1−
2+
2+
2−
3+
3−
4+
4a−
4b−
4b−
5a+
5a-
5+
5−
6+
6−
7+
VII
CrIIa
CrIIb
Par
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Midline
Lateral
PC birthdate compartments (BC)
Embryo (E18.5)
Adult
Zebrin II bands
VII-CrII/Par

Figure 8

following: 1) PC-birth date compartments (BC1–BC8c), 2) zebrin II bands corresponding to each PC birth date compartment, 3) subareas in IO corresponding to each PC birth date compartment, and 4) subareas in the cerebellar nuclei to which PCs in each PC birth date compartment are connected (Sugihara and Quy, 2007). As shown in Table 1, we could determine the pattern of the topographic connection for every PC birth date compartment in the adult cerebellum. These observations highlight the close correlation between the birth date of PCs and the longitudinal compartmentalization revealed by zebrin II immunoreactivity and olivocerebellar/corticonuclear projections.

The close correlation between the birth date of PCs and zebrin II bands also revealed a new aspect of the cerebellar compartmentalization. According to systematic arrangements of each cohort of birth date-related PCs, the cerebellum seems to be divided mediolaterally into three subareas: 1) the medial area that corresponded to the vermis and was composed of BC1, BC2, and BC3 (Fig. 11, medial); 2) the intermediate area that roughly corresponded to the paravermis or pars intermedia and was composed of BC4, BC5, and BC6 (Fig. 11, intermediate); and 3) the lateral area that roughly corresponded to the hemisphere and was composed of BC7, BC8a, and BC8b (Fig. 11, lateral). In the three subareas of VIa-Sim and VII-CrII/Par, PCs born on E10.5 (Fig. 11, green rectangles) were located in the most lateral compartment, PCs born on E12.5 (Fig. 11, red rectangles) were located to more medial compartments, and PCs born on E11.5 (Fig. 11, blue rectangles) were located to the middle compartments. Therefore, PCs formed lateral-to-medial developmental gradients in the three areas [BC3-to-BC1 (Fig. 11, medial), BC6-to-BC5 (Fig. 11, intermediate), and BC8b-to-BC7 (Fig. 11, lateral); Fig. 11, black arrows] in the adult cerebellum.

DISCUSSION

Using an adenoviral vector system, we reveal that cerebellar compartmentalization is determined by the birth date of PCs (Hashimoto and Mikoshiba, 2003). We carefully compared PC birth date compartments in the adult cerebellum with zebrin II bands, which are markers for late-onset patterns and olivocerebellar climbing fiber projections (Buisseret-Delmas and Angaut, 1993; Voogd et al., 2003; Voogd and Ruigrok, 2004; Sugihara and Shinoda, 2004; Sugihara and Quy, 2007). The present study clarifies the relationship between the embryonic compartments (early-onset patterning) and the adult compartments (late-onset patterning). Generally, a single PC birth date compartment in the embryonic cerebellum was directly transformed into a single zebrin II band in the adult. We observed that PC birth date compartments in the adult cerebellum correlated closely with zebrin II bands and therefore also with the organization of the olivocerebellar climbing fiber projections (Table 1). Hence, PC birth date may integrate early- and late-onset patterning with the topographic map of olivocerebellar projections.

PC birth date compartments as the basic units in the cerebellum

To reveal PC birth date compartments, we selected E10.5, E11.5, and E12.5 as the time of the adenoviral injection, because 1) the patterns of compartments formed by PCs born on E10.5, E11.5, and E12.5 (BC1–BC8) are identical to the patterns of early-onset markers, including *Wnt7b* and *En2* (Fig. 1A); 2) each pattern of compartments formed by PCs born on E10.5, E11.5, and E12.5 is unchanged even if adenoviral injection is done 6 hours earlier or 6 hours later than E10.5, E11.5, and E12.5 (data not shown), suggesting that there are no different patterns of compartments formed by the birth date-related PCs between E10.5 and E11.5 or between E11.5 and E12.5; 3) the pattern of PC birth date compartments is stable from embryo to adult; and 4) each pattern of PC birth date compartments is generally constant from mouse to mouse (data not shown). In fact, there was a small variation of β-gal-stained compartments caused by the infectivity of the adenoviral vector. However, the positions of PC birth date compartments were constant. Therefore, we think that the compartments formed by PCs born on E10.5, E11.5, and E12.5 (BC1–BC8) are the basic and immutable units of cerebellar compartmentalization. PCs may acquire their cell fate when they are generated from progenitor cells on the roof of fourth ventricle and form specific subsets of PC birth date compartments in the adult cerebellum according to their distinct characteristics. However, the mechanism by which the fate of

Figure 8. Correlation between PC birth date compartments in the adult cerebellum and zebrin II bands in VII-CrII/Par. **A–O** are arranged in a manner similar to the panels in Figure 6. The boundary between the vermis and hemisphere is indicated by arrows in F,G,L. The diagram in **P** indicates a close correlation between the early-onset pattern represented by PC birth date compartments in the E18.5 cerebellum (upper half) and the late-onset pattern represented by zebrin II immunoreactivity in the adult cerebellum (lower half). The arrows in P show the change from the early-onset pattern to the late-onset pattern. Scale bars = 250 μm.

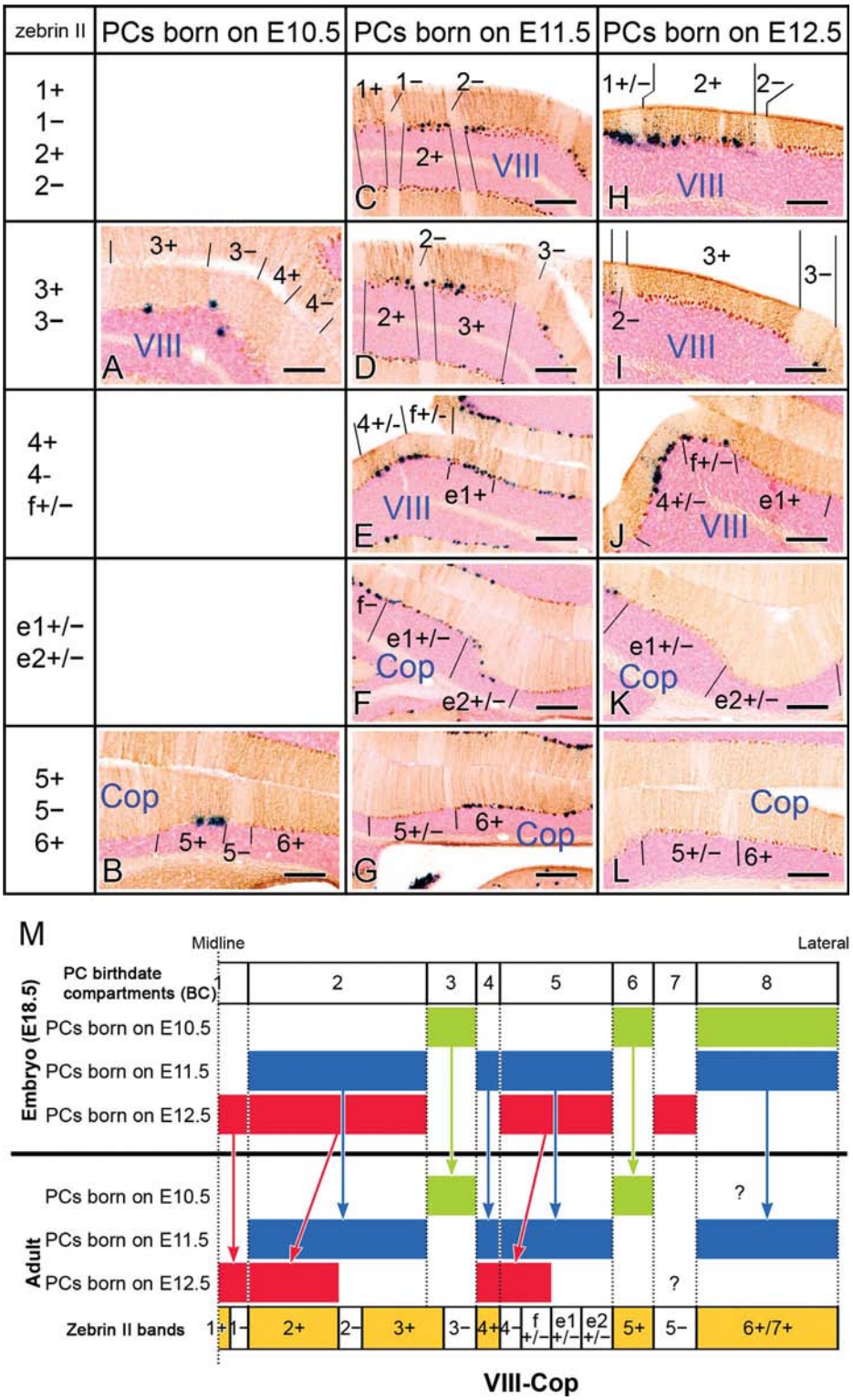


Figure 9. Correlation between PC birth date compartments in the adult cerebellum and zebrin II bands in VIII-Cop. **A–L** are arranged in a manner similar to the panels in Figure 6. The diagram in **M** indicates a close correlation between the early-onset pattern represented by PC birth date compartments in the E18.5 cerebellum (upper half) and the late-onset pattern represented by zebrin II immunoreactivity in the adult cerebellum (lower half). The arrows in M show the change from the early-onset pattern to the late-onset pattern. PC birth date compartment (BC3) was not identical to zebrin bands (3+ and 3–). We could not detect BC7, which might correspond to 5– (question mark), or BC8, which arises at E10.5, in the adult cerebellum. Scale bars = 250 μm.

TABLE 1.

Close Correlations Among PC Birth Date Compartments, Zebrin II Bands, Olivocerebellar Projection, and Corticonuclear Projection in the Adult Cerebellum

	PC-birthdate compartments[1]	Birthdate of PC[1]	Cerebellar cortex (Rostral → Caudal) Zebrin II-band[2] IV-V	VIa-Sim	VII-CrII/Par	VIII-Cop	IX	Target nucleus of PC[3]	Olivo-cerebellar Zone[4]	Origin of olivocerebellar projection: Subnucleus of IO[5]	Subarea[5]	
Medial	BC1	E12.5	1+	1+	1+	1+	1+	MCN	A	cMAO-a	Caudal	
			—	1–	1–	1–	1–	MCN	A	cMAO-b	Lateral	Caudal
	BC2	E11.5/ E12.5	—	a+ (E11.5/E12.5)	2+ (E11.5/E12.5)	2+ (E11.5/E12.5)	2+ (E11.5/E12.5)	MCN	A	Beta cMAO-c	Caudal Medial	
			1– (E11.5/E12.5)	a– (E11.5)	2– (E11.5)	2– (E11.5)	2– (E11.5)	MCN	A	cMAO-b	Lateral	Intermediate
			2+ (E11.5)	2+ (E11.5)	3+ (E11.5)	Medial half of 3+ (E11.5)	3+ (E11.5)	MCN/ICG	A (AX)	cMAO-a	Intermediate and Rostral	
	BC3	E10.5	—	—	—	Lateral half of 3+	—	ICG	A (AX)	cMAO-a	Intermediate and Rostral	
			medial part of 2–	2–	3–	3–	3–	ICG	X	cMAO-b	Lateral	Rostral
Intermediate	BC4	E11.5	b+	2b+	4+	4+	4+	PIN	X-CX	DMCC, caudal DM, caudomedial vPO		
	BC5	E11.5/ E12.5	—	2b– (E12.5)	4a– (E12.5)	—	—	DLP	A2	cMAO-b	Medial	Caudolateral
			—	c+ (E11.5)	4b+ (E11.5/E12.5)	—	—	DLP	A2	cMAO-c	Lateral	Caudal
			—	c– (E11.5)	4b– (E11.5/E12.5)	—	—	DLP	A2	cMAO-b	Medial	Caudomedial
			—	d+ (E11.5)	5a+ (E11.5)	—	—	DLP	A2	cMAO-c	Lateral	Rostral
			—	d– (E11.5)	5a– (E11.5)	—	—	DLP	(Lateral A2)	cMAO-b	Medial	Intermediate
			(2–)	—	—	4– (E11.5/E12.5)	—	LVN	B	dDAO		
			(b+) b– (E12.5) 3+ (E11.5) 3– (E11.5/E12.5)	—	—	f+ (E11.5/E12.5) f– (E11.5/E12.5) e1+/– (E11.5) e2+ (E11.5)	—	AIN	C1	vDAO	Caudolateral	
			(3–)	—	—	? (e2–)	—	PIN	CX	cMAO-b	Medial	Rostral
	BC6	E10.5	4+	4+	5+	5+	—	PIN	C2	rMAO		
Lateral	BC7	E12.5	?(4–)	4–	5–	? (5–)	—	AIN	C3	vDAO	Rostromedial	
	BC8 BC8a	E11.5	?	5+	6+	6+	—	LCN	D1	vPO		
	BC8b	E10.5	?	5–	6–	–	—	DLH	D0	DM	Central and rostral	
	BC8c	E11.5	?	6+	7+	7+	—	LCN	D2	dPO		

[1]The compartments formed by birthdate-related Purkinje cells (Hashimoto and Mikoshiba, 2003). See Results for details.
[2]Names and positions of zebrin II-bands were determined according to Sughihara and Shinoda (2004) and Sugihara and Quy (2007). See Results for details.
[3]According to Buisseret-Delmas and Angaut (1993), Voogd and Ruigrok (2004), Sugihara et al., (2009)
[4]According to Voogd, et al., (2003), Voogd and Ruigrok (2004), and Sugihara and Quy (2007).
[5]According to Sugihara and Quy (2007).The principal olive (PO) is further subdivided into the ventral (vPO) and dorsal lamellas (dPO), and the dorsomedial group subnucleus (DM). The dorsal accessory olive (DAO) is divided into dorsal (dDAO) and ventral folds (vDAO). The medial accessory olive (MAO) is divided into rostral (rMAO) and caudal parts (cMAO). The cMAO is further subdivided cytologically into lateral (cMAO-a), intermediate (cMAO-b), and medial parts (cMAO-c). AIN, anterior interposed nucleus; Beta, subnucleus beta; DLH, dorsolateral hump; DLP, dorsolateral protuberance; DMCC, dorsomedial cell column; ICG, interstitial cell group; LCN, lateral cerebellar nucleus; LVN, lateral vestibular nucleus; MCN, medial cerebellar nucleus; PIN, posterior interposed nucleus.

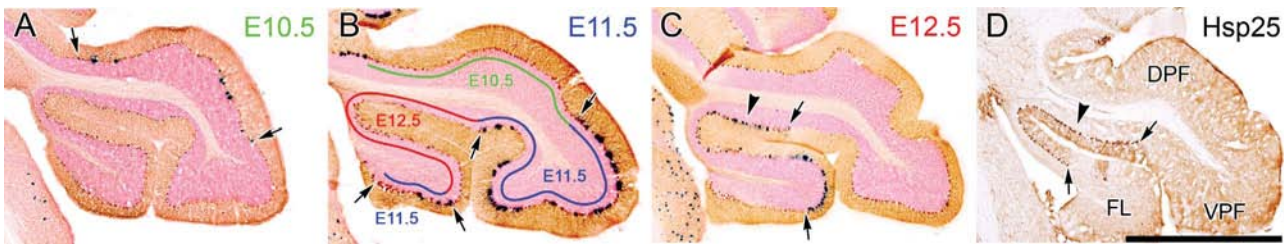


Figure 10. PC birth date compartments in PF and FL. The sections of PF and FL (**A–C**) were stained for β-gal (dark blue or black color), immunostained with antizebrin II antibody (blown color), and counterstained with Nuclear Fast Red (pink color). PF and FL are clearly compartmentalized into several regions by PCs born on E10.5 (A), E11.5 (B), and E12.5 (C). The borders of each compartment are indicated by arrows in A–C. The schematic drawing in B represents the regions of compartments formed by PCs born on E10.5 (green line), E11.5 (blue lines), and E12.5 (red line). The expression of zebrin II does not reveal compartments in PF and FL because all PCs in PF and FL are zebrin II positive (A–C). In contrast, the Hsp25 immunoreactivity (**D**) shows a compartment in the ventromedial region of PF and the dorsomedial region of FL. The borders of the Hsp25-immunopositive region are indicated by arrows (D). Arrowheads indicate the ventromedial region of PF. Scale bar = 1 mm.

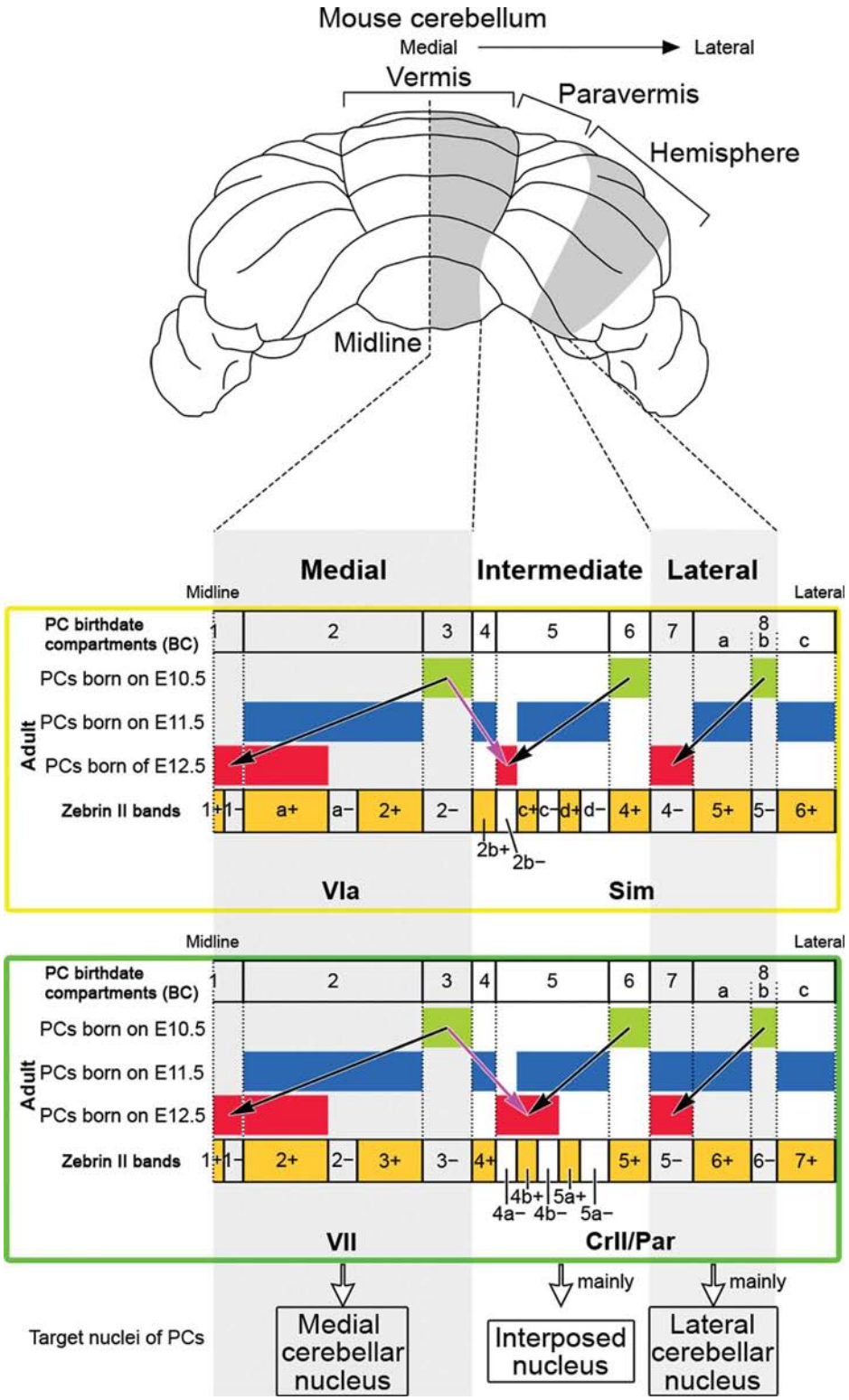


Figure 11. Three units of a lateral-to-medial developmental gradient in the adult cerebellum. The correlation between PC birth date compartments in the adult cerebellum and zebrin II bands in VIa-Sim (upper half) and in VII-CrII/Par (lower half) are indicated. There are three units of a lateral-to-medial (E10.5 to E12.5) developmental gradient in the cerebellum (black arrows). These correspond to the vermis (medial), medial half of hemisphere (intermediate), and lateral half of hemisphere (lateral). The boundary between the vermis and hemisphere is located between BC3 and BC4 (2− and 2b+ in VIa-Sim; 3− and 4+ of in VII-CrII/Par). In contrast to the three lateral-to-medial developmental gradient units (black arrows), BC3, BC4, and the medial half of BC5 formed a medial-to-lateral developmental gradient (magenta arrows).

PCs is determined and the characteristics of each birth date-related PC are still unclear.

Interrelationship between early-onset and late-onset patterning

Several studies have compared the embryonic and adult compartments by mapping the expression of molecules in early postnatal periods (Larouche and Hawkes, 2006; Marzban et al., 2007) or in a transgenic animal (Sillitoe et al., 2009). Although these studies have demonstrated that some multiple late-onset compartments originated from a single early-onset compartment, the relationship between the patterns of these two compartments has not been thoroughly clarified. Here, we systematically examined the relationship between PC birth date compartments in the embryonic cerebellum (early-onset patterning) and zebrin II bands (late-onset patterning). In general, a single PC birth date compartment (group 1 type: BC1, BC3, BC4, BC6, and BC7) in the embryonic cerebellum (early-onset pattern) was directly transformed into a single zebrin II band in the adult cerebellum (late-onset pattern; Table 1). In contrast, the formation of BC2, BC5, and BC8 (group 2 type) was more complex than the other PC birth date compartments (group 1 type). BC2, BC5, and BC8 were each formed by two different birth cohorts of PCs (Figs. 6N, 7M, 8P, 9M). BC2 and BC5 were formed by PCs born on E11.5 and E12.5, whereas BC8 was formed by PCs born on E10.5 and E11.5. This overlap of different PC birth cohorts contributes to the complexity of the late-onset patterning. In BC2 and BC5, PCs born on E12.5 were located to the more medial region than PCs born on E11.5 (Figs. 6N, 7M, 8P, 9M, 11). Therefore, PC cohorts born on E11.5 and E12.5 in BC2 and BC5 divided these regions into narrower bands of zebrin II-positive and -negative bands. Similarly, the distribution of PCs born on E10.5 and E11.5 in BC8 generates three subcompartments (BC8a, BC8b, and BC8c) in the adult cerebellum. The relocation of the birth date-related PCs contributed to the positioning of zebrin II bands in BC2, BC5, and BC8. Therefore, our observation suggests that PC birth date compartments in the embryonic cerebellum (early-onset patterning) are basic units of the establishment of M-L compartments in the adult cerebellum defined by zebrin II bands (late-onset patterning). Furthermore, PC birth date compartments were identical to the topographic pattern of olivocerebellar climbing fiber projections (Table 1). Each PC birth date compartment seems to provide different positional information to the circumstances (e.g., Eph-ephrin signaling; Nishida et al., 2002) and be concerned with the establishment of the topographic pattern of olivocerebellar projections. However, the molecular mechanisms by which the cerebellar compartmentalization is established are not clear.

In VIb/c-CrIb (Fig. 2, white dotted line), lobule X (Fig. 2, X), FL (Fig. 2, FL), and PF (Fig. 2, PF), there are no zebrin II-negative bands, because all PCs are immunopositive for zebrin II (for reviews see Sillitoe and Joyner, 2007; Apps and Hawkes, 2009). Therefore, it seems that there are no cerebellar compartments in these regions. However, the immunoreactivity of anti-Hsp25 reveals another longitudinal compartments in VIb/c, lobule X, PF, and FL (Armstrong et al., 2000; Sillitoe and Joyner, 2007; Sillitoe et al., 2008; Apps and Hawkes, 2009). Furthermore, olivocerebellar projections clearly form zonal compartments in these regions (Ruigrok, 2003; Sugihara and Shinoda, 2004; Voogd and Ruigrok, 2004; Sugihara et al., 2004; Schonewille et al., 2006), and the Hsp25 immunoreactivity is correlated with the topographic map of olivocerebellar projections and physiological functions (Schonewille et al., 2006). Similarly, the birth date-related PCs formed longitudinal compartments in VIb/c-CrIb and lobule X (Figs. 3A,E, 4), and PF and FL (Fig. 1H,I, arrowheads, Figs. 4, 10A–C). Therefore, it is difficult to examine the relationship between PC birth date compartments and zebrin II bands in VIb/c-CrIb, lobule X, PF, and FL, but PC birth date compartments can be compared with Hsp-25 immunoreactivity in these regions. The comparison between PC birth date compartments and Hsp25 immunoreactivity (e.g., Fig. 10) is helpful for understanding the relationship among PC birth date compartments, the topographic map of olivocerebellar projections, and physiological functions in these regions. Interestingly, the compartment formed by PCs born on E12.5 in PF and FL seems to agree with the Hsp25-immunopositive compartment (Fig. 10C,D). The observation suggests that PC birth date compartments in PF and FL are formed independently of zebrin II immunoreactivity and are correlated with the topographic map of olivocerebellar projections in PF and FL.

It has been reported that climbing and mossy fibers have collateral branches that are projected into separate cerebellar lobules (Voogd et al., 2003; Ruigrok, 2003; Pijpers et al., 2006). For instance, climbing fibers that are projected to zebrin II band 4– in VIa-Sim and 5– in VII-CrII/Par send their collateral branches into zebrin II 5a– in VII-CrII/Par. The collateralization of climbing fibers suggests a functional relationship among the separate longitudinal compartments. IO neurons in the rostral part of medial accessory olive (rMAO) project their climbing fibers to zebrin II-positive regions (4+ in IV-V and VIa-Sim, 5+ in VII-CrII/Par and VIII-Cop), and their collateral branches are projected to the caudal half of PF (Buisseret-Delmas and Angaut, 1993; Sugihara et al., 2004; Pijpers et al., 2005; C2 olivocerebellar zone). The

compartments formed by PCs born on E10.5 [4+ in IV-V and VIa-Sim, 5+ in VII-CrII/Par and VIII-Cop (Table 1, BC6); caudal half of PF (Fig. 1H, arrowhead; Fig. 4A, PF)] were identical to the target regions of rMAO. This coincidence suggests that there is a correlation between the collateralization of climbing fiber projection and the PC birth date compartments.

Developmental gradient in the cerebellum

Results of the present study indicate that the cerebellum is composed of three zones represented by lateral-to-medial developmental gradients of PCs [BC3-to-BC1 (Fig. 11, medial), BC6-to-BC5 (Fig. 11, intermediate), BC8b-to-BC7 (Fig. 11, lateral); Fig. 11, black arrows]. The earliest born (E10.5) PCs are located near the lateral edge (BC3, BC6, BC8b) in each of the three zones (Fig. 11, green rectangles). PCs that were born on the following day (E11.5) are located in more medial regions (Fig. 11, blue rectangles) than the compartment formed by PCs born on E10.5. The latest born (E12.5) PCs settle in the most medial regions (Fig. 11, red rectangles). It is likely that the generation of the three earliest PC birth date compartments (BC3, BC6, BC8b) underlies the three zonal organization of the cerebellum. This is the first observation of a distinct developmental gradient in the cerebellum. It is known that PCs located in vermis, paravermis, and hemisphere preferentially project their axons to the medial cerebellar, interposed, and lateral cerebellar nuclei, respectively (Goodman et al., 1963; Brodal, 1981; Paxinos and Franklin, 2003; Voogd and Ruigrok, 2004; Apps and Garwicz, 2005; Sugihara et al., 2009; refer to Fig. 11, target nuclei of PCs; Table 1, target nucleus of PC). Therefore, the cerebellum is generally divided into three regions (vermis, paravermis, and hemisphere) according to the corticonuclear projections. Interestingly, three zones represented by the lateral-to-medial developmental gradients of PCs (Fig. 11, medial, intermediate, lateral) were identical to vermis, paravermis, and hemisphere of the cerebellum (Fig. 11, Table 1). The observation suggests that there is a correlation between three zones represented by a lateral-to-medial developmental gradient of PCs and the pattern of cerebellar corticonuclear projection. We hypothesize that PC birth date compartments contribute to the establishment of the topographic map of corticonuclear projections in the cerebellum, but further studies are necessary to confirm this.

In contrast to the three zones, a zone from BC3 to the medial BC5 in VIa-Sim and VII-CrII/Par formed a medial-to-lateral developmental gradient of PCs (Fig. 11, magenta arrows). The boundary between the vermis and paravermis seemed to be located between BC3 and the medial BC5 in VIa-Sim (Fig. 7D,E,I, arrows) and VII-CrII/Par (Fig. 8A,F,L, arrows). Therefore, we assume that the zone from BC3 to the medial BC5 (Fig. 11, magenta arrows) probably plays an important role in the definition of the boundary between the vermis and the paravermis. However, further analysis is required to explore this.

Physiological significance of PC birth date compartments in the adult cerebellum

Neuronal activity is often longitudinally organized in the cerebellar cortex, as seen in optical recordings from the cortical surface (Gao et al., 2006), spontaneous complex spike activity in multiple PCs (Sugihara et al., 2007), and evoked responses by peripheral stimulation (Oscarsson and Sjolund, 1977). As a basis of these functional organizations, projection patterns of afferent and efferent axons are closely correlated to the longitudinal compartments that are visualized by zebrin II immunostaining (Buisseret-Delmas and Angaut, 1993; Voogd et al., 2003; Sugihara and Shinoda, 2004; Sugihara and Quy, 2007; Pijpers et al., 2006; Sugihara et al., 2009). In addition, the expression of an excitatory amino acid transporter subtype 4 (EAAT4; Dehnes et al., 1998; Wadiche and Jahr, 2005) and phospholipase Cβ4 (Kano et al., 1998; Sarna et al., 2006), which directly regulate cerebellar activities, are closely correlated with zebrin II bands. Furthermore, recent studies (Voogd et al., 2003; Ruigrok, 2003; Pijpers et al., 2006) indicate that the topographic organization of mossy fiber inputs is closely correlated with zebrin II bands and the topographic map of climbing fiber inputs in the adult rat cerebellum. Therefore, the neuronal activity and the neuronal network of the cerebellum are highly organized according to the longitudinal compartments in the cerebellum. Our observations indicated the close correlation between PC birth date compartments (early-onset pattern) and zebrin II bands (late-onset pattern), and therefore also the topographic map of olivocerebellar/corticonuclear projections (Table 1). Furthermore, PC birth date compartments are probably concerned with the topographic organization of mossy fiber inputs (Voogd et al., 2003; Ruigrok, 2003; Pijpers et al., 2006). Gross axonal projection in the cerebellum is generally established much earlier during embryonic development than establishment of the late-onset pattern (Leclerc et al., 1988; Wassef et al., 1990; Seil et al., 1995; Gallagher et al., 1998; Marzban et al., 2007). This suggests that PC birth date compartments (early-onset pattern) play an important role in establishing the cerebellar neuronal network as well as in forming the late-onset pattern.

Neuronal activity recorded by flavoprotein autofluorescence in vivo imaging in the adult mouse cerebellum (Gao et al., 2006) may be related to PC birth date compartments in the cerebellum. We have demonstrated three

bands along the parasagittal axis, b1, b2, and b3, in CrII after parallel fiber stimulation and, furthermore, two bands, b1 and the region between b2 and b3, after peripheral stimulation of the vibrissal pad. This indicates that b1, b2, the region between b2 and b3, and b3 correspond to zebrin II bands 5+ (the original paper called this region 5b+), 6+, 6–, and perhaps 7+, respectively (Table 1). Our observations show that these bands are identical to BC6, BC8a, BC8b, and BC8c in CrII, respectively. Among these bands the two that receive the peripheral inputs, b1 and the region between b2 and b3, corresponded to BC6 and BC8b, both of which arise from PCs born on E10.5 (Fig. 8P, Table 1). Although the mechanism of how these two bands are activated by peripheral stimulation is not clear, the observations suggest that the adult longitudinal compartments formed by a PC cohort that shares the same birth date might contribute to cerebellar function. The results of the present study (Table 1) allow us to study the relationship between PC birth date compartments and neuronal activity in the cerebellum. A further study will reveal the relationship between PC birth date compartments and functional compartments in the cerebellum.

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