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REGULAR ARTICLE

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Granule cell raphes in the developing mouse cerebellum

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Abstract The cerebellar cortex of many vertebrates shows a striking parasagittal compartmentation that is thought to play a role in the establishment and maintenance of functional cerebellar connectivity. Here, we demonstrate the existence of multiple parasagittal raphes of cells in the molecular layer of the developing cerebellar cortex of postnatal mouse. The histological appearance and immunostaining profile of the raphe cells suggest that they are migrating granule cells. We therefore conclude that the granule cell raphes previously described in birds also exist in a mammalian species. The raphes in mouse are visible on nuclear stains from around birth to postnatal day 6 and are frequently found at the boundaries of Purkinje cell segments that differentially express cadherins ("early-onset" parasagittal banding pattern). A similar relation between the raphe pattern and various markers for the early-onset banding pattern has been found in the chicken cerebellum. One of the cadherins mapped in the present study (OL-protocadherin) continues to be expressed in specific Purkinje cell segments until at least postnatal day 14. At this stage of development, the borders of the OL-protocadherin-positive Purkinje cell segments coincide with the borders of Purkinje cell segments that express zebrin II, a marker for the "late-onset" parasagittal banding pattern which persists in the adult cerebellum. These findings demonstrate that the early-onset banding pattern, as reflected in the complementary arrangement of raphes/Purkinje cell segments, and the late-onset pattern of zebrin II expression share at least some positional cues during development.

Keywords Pattern formation · Migration · Purkinje cells · Cell adhesion molecules · Cadherin · Mouse (C57BL)

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Introduction

In many vertebrate species, the cerebellar cortex shows a striking parasagittal modular organization (for reviews, see Hawkes and Mascher 1994; Herrup and Kuemerle 1997; Oberdick et al. 1998), which is reflected not only in its afferent and efferent connectivity (Groenewegen and Voogd 1976; Arends and Zeigler 1991; Wassef et al. 1992; Voogd and Glickstein 1998) but also in the expression of various molecular markers such as gene regulatory proteins (see Millen et al. 1995; Lin and Cepko 1998), cell adhesion molecules (see Arndt and Redies 1996; Chédotal et al. 1996; Fushimi et al. 1997; Korematsu and Redies 1997a; Suzuki et al. 1997; Arndt et al. 1998; for review, see Redies 2000), and other types of molecules (see Wassef et al. 1985; Eisenman and Hawkes 1993; Oberdick et al. 1993). It is generally agreed upon in the literature that these molecules can be grouped into markers that reflect an "early-onset" parasagittal banding pattern in the developing (perinatal) cerebellum and those that reflect a "late-onset" banding pattern that sets in later in development and persists in the mature cerebellum (for review, see Herrup and Kuemerle 1997). The relationship between these two types of patterns has remained elusive, because there is, in general, no or little temporal overlap between the expression of early-onset markers and late-onset markers in the parasagittal compartments. While some authors have speculated that the two types of pattern are related to one another (see Herrup and Kuemerle 1997), a recent study has shown that the two types of pattern are differentially affected by the ectopic expression of the gene regulatory molecule En2 (Baader et al. 1999). The precise number of mediolateral compartments is also a matter of controversy (for review, see Oberdick et al. 1998). On the one hand, it is clear that a vast number of unique domains can be defined based on their expression of combinations of the parasagittal markers available to date. On the other hand, heterogeneities and borders of expression are often found to coincide for several different markers. Based on an analysis of such common borders, it has been proposed that there exist a set of about

six to eight distinct mediolateral compartments (for review, see Herrup and Kuemerle 1997).

We have recently shown that, during the early-onset phase of parasagittal patterning in the chicken, the cerebellar cortex contains parasagittal ribbons of migrating granule cells and interneurons that express specific cadherins. These ribbons of migrating cells extend from the external granular layer through the developing molecular layer into the internal granular layer. We proposed (Fushimi et al. 1997; Arndt et al. 1998) that the ribbons are identical to the "granule cell raphes" first described on histological grounds by Feirabend (1983) in chicken between approx. 9 and 15 days of incubation. The granule cell raphes separate parasagittal segments of Purkinje cells that differentially express cadherins (Arndt et al. 1998). This complementary arrangement has been confirmed by Lin and Cepko (1998), who showed that several other molecules also exhibit expression domains that coincide with the pattern of granule cell raphes.

In the present study, we show that granule cell raphes are also found in the cerebellar cortex of the mouse. As in the chicken, these granule cell raphes show a higher cell density than the surrounding molecular layer, and they are frequently found at the borders of cadherin-expressing Purkinje cell clusters. Note that the term "raphe" has been previously used by Korneliussen (1967, 1968) for cell-poor ("medullary") structures observed between longitudinal zones of developing cerebellar cortex and deep cerebellar nuclei of several mammalian species. These structures are different from the cell-dense granule cell raphes described in the present work. To our knowledge, granule cell raphes (in the sense used by Feirabend, 1983) have not been described in any nonavian species to date.

Moreover, one of the cadherins examined in the present study (OL-protocadherin; Hirano et al. 1999) is continuously expressed in distinct cell clusters from embryonic day 15 (E15) to at least postnatal day 14 (P14). This marker allowed us to relate specific topological positions within the early-onset parasagittal patterning to heterogeneities in the expression pattern of a well-established marker for the late-onset pattern (zebrin II; Eisenman and Hawkes 1993).

Materials and methods

Animals

Pups from C57BL mice were deeply anesthetized on ice and decapitated. Staged pregnant C57BL mice were deeply anesthetized by inhalation of diethyl ether and killed by cervical dislocation. These procedures were in accordance with the current version of the German Law on the Protection of Animals and institutional guidelines on the use of animals in research. Brains were dissected and fixed in 4% formaldehyde dissolved in HEPES-buffered salt solution supplemented by 1 mM Ca^{2+} and 1 mM Mg^{2+} (HBSS). Following cryoprotection in 12%, 15%, and 18% sucrose in HBSS, brains were mounted in Tissue-Tek OCT compound (Sakura Finetek, Torrance, Calif., USA) and frozen in liquid nitrogen. The following stages were used: E16, E18, P0, P2, P3, P4, P5, P7, P10, and P14. The day of birth was designated as P0. All animals were obtained from the animal facilities at the University of Essen School of Medicine.

In situ hybridization

A previously published protocol was followed (Redies and Takeichi 1993; Gänzler and Redies 1995). Digoxigenin-labeled antisense cRNA probes were produced by in vitro translation from the following vectors: mcad8–12 containing a 1.6-kb fragment of mouse cadherin-8 cDNA from the 5′ region (Korematsu and Redies 1997b); pSP73 containing the 2.5-kb *Sma*I-*Pst*I fragment of mouse cadherin-11 cDNA (kind gift of Drs. Y. Kimura and M. Takeichi, Kyoto University; Kimura et al. 1995); pBSMR4 containing full-length mouse R-cadherin cDNA (kind gift of Drs. H. Matsunami and M. Takeichi; Matsunami and Takeichi 1995); and Mpr-655 containing the *Hin*dIII-*Xba*I fragment of mouse TAG-1 cDNA (kind gift of Dr. S. Kozlov, University of Zurich; Wolfer et al. 1998). Briefly, in situ hybridization was performed on 20-µm-thick cryosections mounted on coated slide glasses. The sections were fixed in 4% formaldehyde/HBSS, permeabilized by treatment with proteinase K solution (1 µg/ml), postfixed in 4% formaldehyde/HBSS, and treated with acetic anhydride solution. After overnight hybridization with the cRNA probes, sections were washed and treated with RNase (20 µg/ml in NTE buffer). Digoxigenin-labeled cRNAs were detected with alkaline phosphatase-conjugated Fab fragments against digoxigenin (Boehringer, Mannheim, Germany), followed by a coloring reaction using X-phosphate and nitroblue tetrazolium salt as substrates. Sections were either directly dehydrated and mounted in Entellan (Merck, Darmstadt, Germany) or counterstained for nuclei with ethidium bromide (1 µg/ml in TRIS-buffered saline) and mounted in Vectashield medium (Vector Laboratories, Burlingame, Calif., USA).

Immunohistochemistry

Procedures were carried out as described previously (Gänzler and Redies 1995; Arndt et al. 1998). The following primary antibodies were used: mouse monoclonal antibody against chicken calbindin-D (clone CL-300; Sigma, Deisenhofen, Germany), mouse monoclonal antibody zebrin II against aldolase C (kind gift of R. Hawkes, University of Calgary; Brochu et al. 1990; Ahn et al. 1994), and mouse monoclonal antibody 4D7/TAG-1 against rat TAG-1, developed by M. Yamamoto, obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, under contract NO1-HD-7-3263 from the NICHD (Dodd et al. 1988). Rat monoclonal antibody 5G10 against a GST fusion protein of the cytoplasmic domain of an isoform of OL-protocadherin (S. Hirano, S. T. Suzuki, unpublished observations) was obtained as described previously (Hirano et al. 1999).

For detection of primary antibody, appropriate secondary antibodies labeled with the indocarbocyanine dye Cy3 or with peroxidase were used. The peroxidase-labeled antibodies were detected using a commercially available kit (Vector ABC Elite kit, Vector Laboratories) and were counterstained for nuclei with ethidium bromide. Sections stained with fluorescent secondary antibodies were counterstained with nuclear dye Hoechst 33258 (Sigma). Sections were mounted with Vectashield medium. For several series of sections, adjacent sections were stained for Nissl substance with thionine acetate solution. All sections were viewed and photographed under a light-transmission microscope equipped for epifluorescence detection (Axioplan, Zeiss, Oberkochen). Processing of scanned images was carried out on a computer using the Photoshop program (Adobe, Mountain View, Calif.).

For a reconstruction of calbindin immunoreactivity in P5 cerebellum, a complete series of sections spaced 40 µm apart was obtained and counterstained with nuclear dye (ethidium bromide). Immunostaining of all sections was photographed with a digital camera. The position of all gaps of immunoreactivity in the prospective Purkinje cell layer and/or of nuclear ribbons in the molecular layer (see Results section) was detected through the microscope at high magnification and marked on the digitized sections. After an identification of the cerebellar lobules in each section, the position of the gaps was transferred onto anterior- and posterior-

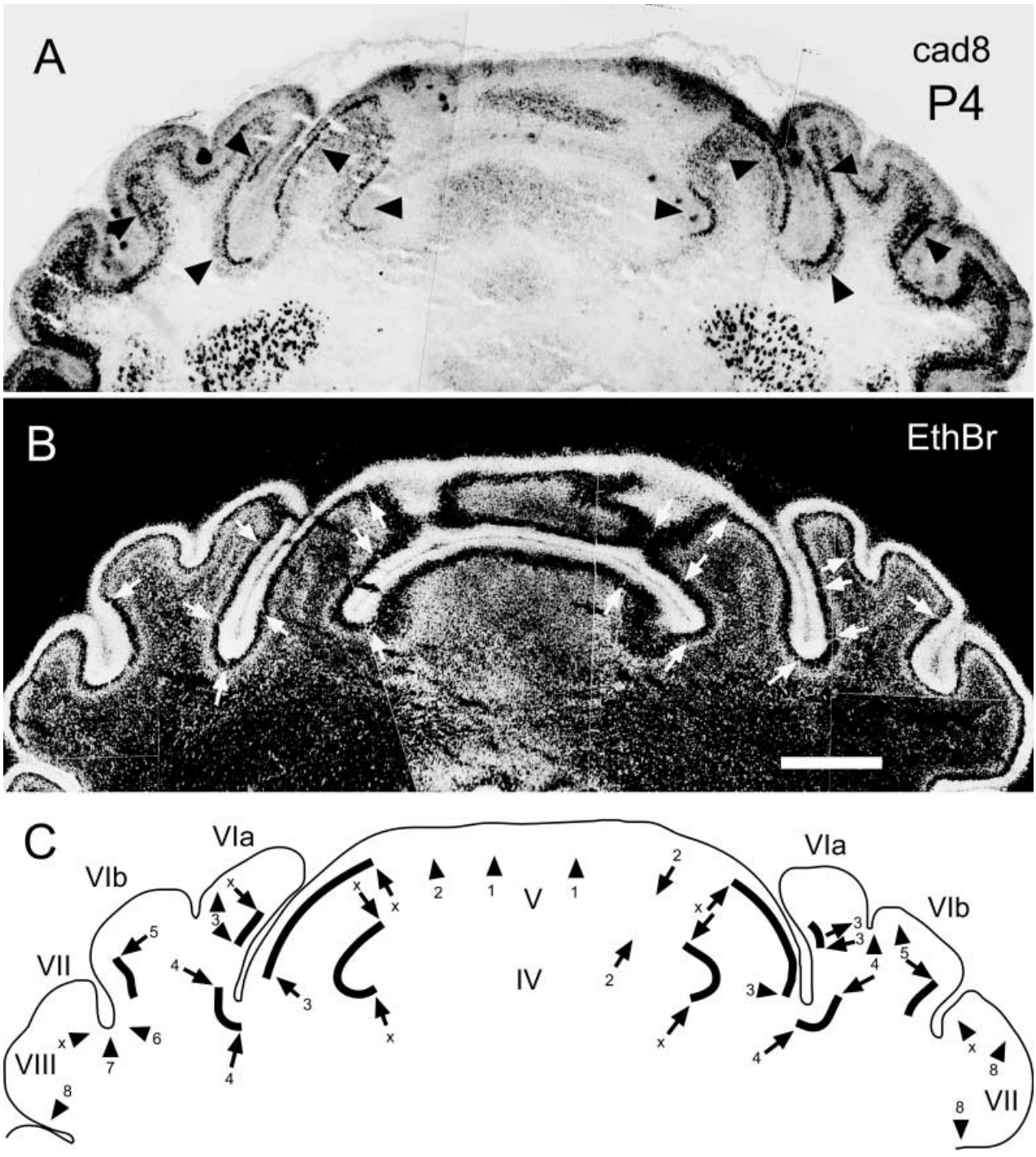
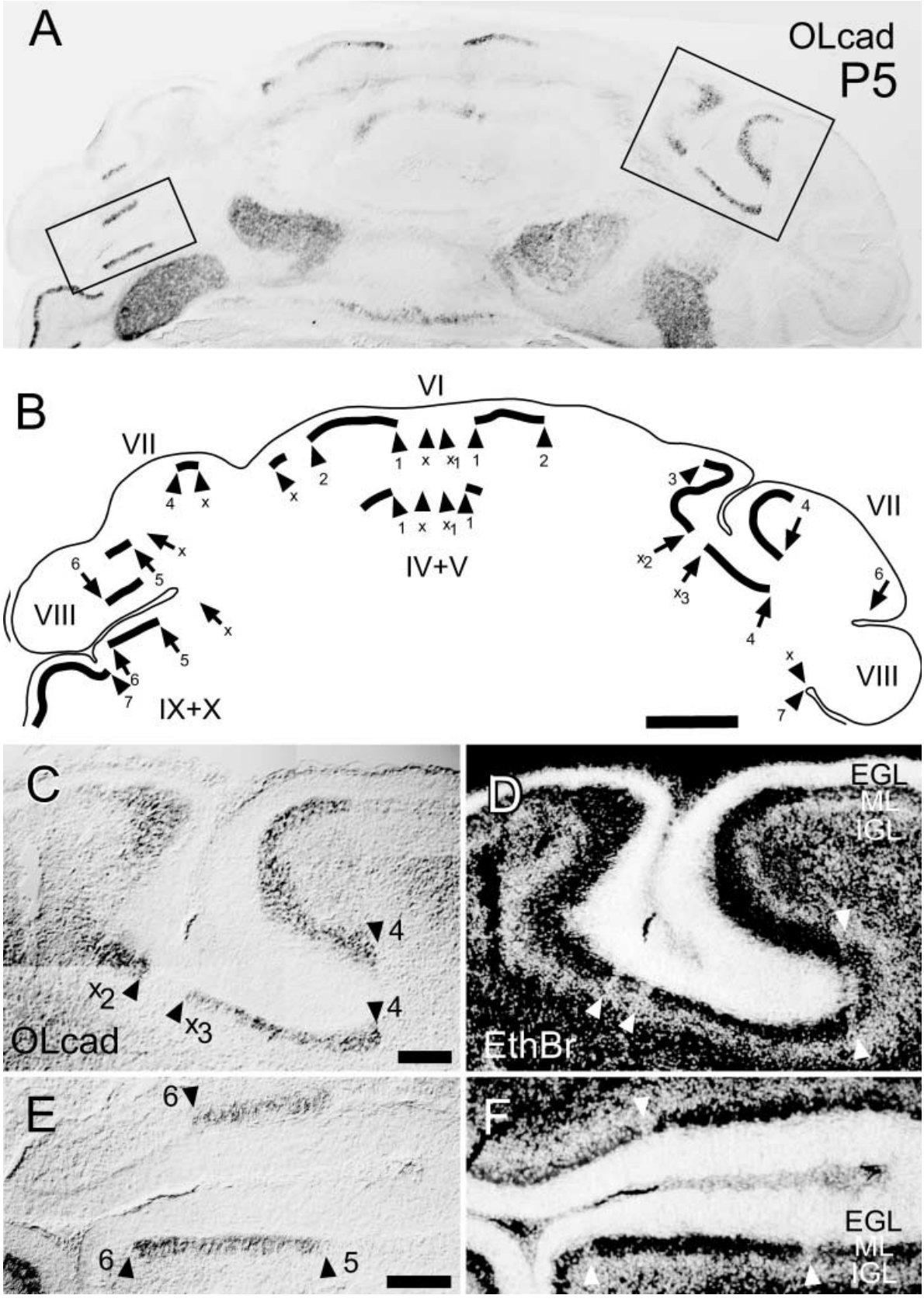


Fig. 1A–C Spatial relationship between cadherin-8-expressing Purkinje cell clusters and ribbons of cell nuclei (raphes) in the molecular layer. A transverse section through a P4 mouse cerebellum was hybridized in situ with antisense probe for cadherin-8 (*cad8* in **A**) and counterstained for cell nuclei with ethidium bromide (*EthBr* in **B**). The schematic drawing in **C** shows the spatial relationship between the cadherin-8-positive Purkinje cell clusters (*arrowheads* in **A**, *thick lines* in **C**) and the nuclear ribbons (*arrows* in **B, C**). The *arrowheads* in **C** indicate the position of ribbons that were detected in immediately adjacent sections. *Arabic numerals* mark individual ribbons that extend over almost all lobules, as depicted in Fig. 6. The letter *x* indicates the positions of additional ribbons. *Roman numerals* mark the cerebellar lobules. *Bar* 500 µm

angled schematic views of P5 cerebellum (modified after color Figs. 4B and 5B by Altman and Bayer, 1997).

Results

Figure 1 shows a complete transverse section through the postnatal day 4 (P4) mouse cerebellum hybridized in situ with antisense probe for cadherin-8 (Fig. 1A) and counterstained with a nuclear dye (Fig. 1B). A similar transverse section through P5 cerebellum immunostained for OL-protocadherin is shown in Fig. 2A. As demonstrated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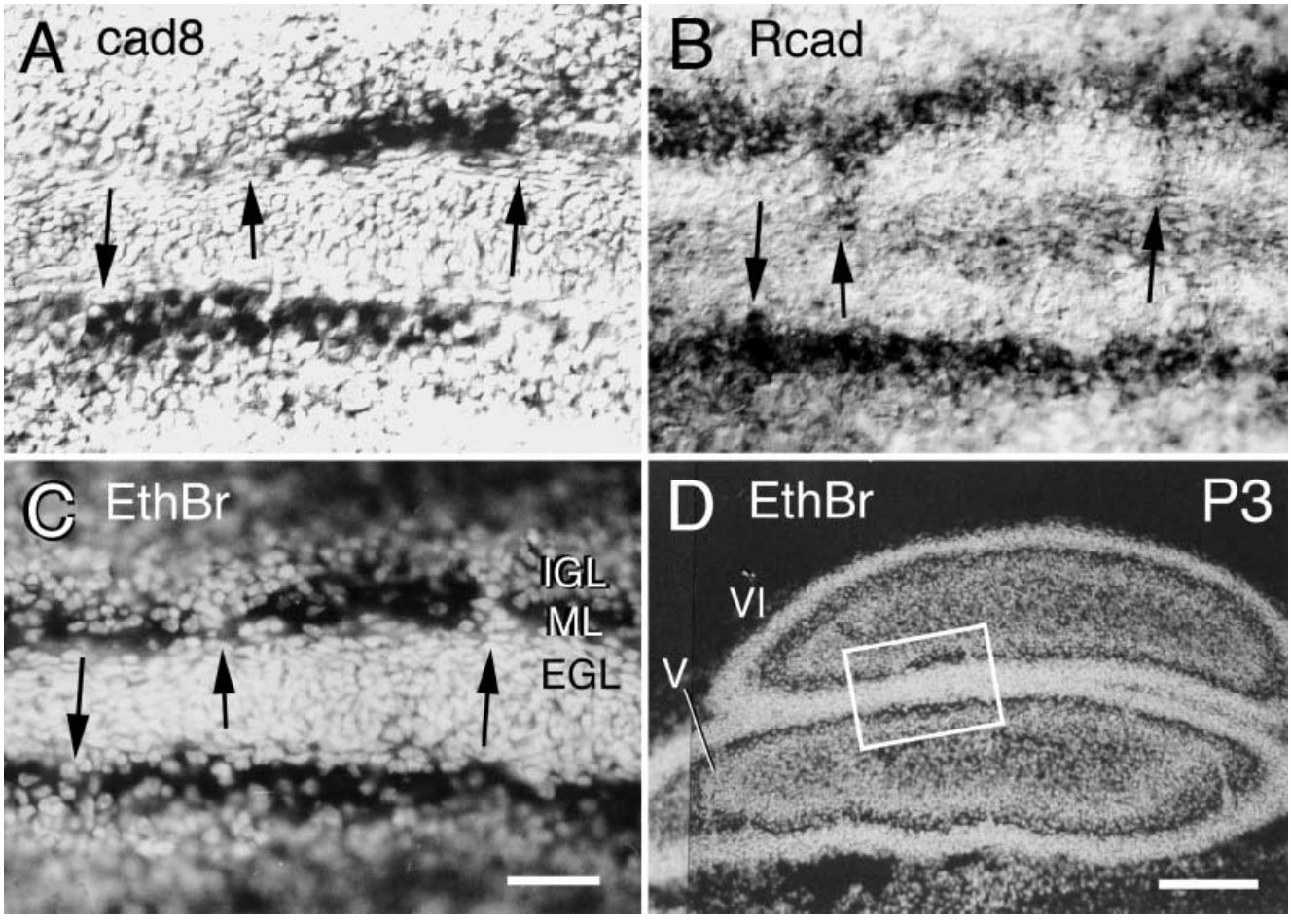

Fig. 3A–D Cadherin-8-expressing Purkinje cell clusters bordered by R-cadherin-expressing nuclear ribbons (raphes) in the molecular layer. Adjacent transverse sections through a P3 mouse cerebellum were hybridized in situ with antisense probes for cadherin-8 (*cad8* in **A**) and R-cadherin (*Rcad* in **B**). The section shown in **A** was counterstained for cell nuclei with ethidium bromide (*EthBr* in **C, D**). The *arrows* in **A**–**C** indicate the position of the nuclear ribbons. **A**–**C** show enlargements of the area *boxed* in **D**. *Roman numerals* mark the cerebellar lobules (*IGL* internal granular layer, *EGL* external granular layer, *ML* molecular layer). *Bars* **A–C** 50 µm; **D** 200 µm

◀ **Fig. 2A–F** Spatial relationship between OL-protocadherin-positive Purkinje cell clusters and ribbons of cell nuclei (raphes) in the molecular layer. A transverse section through a P5 mouse cerebellum was immunostained with antibodies against OL-protocadherin (*OLcad* in **A**) and counterstained with the nuclear dye ethidium bromide (data not shown). The schematic drawing in **B** shows the spatial relationship between the OL-protocadherin-positive Purkinje cell clusters (*thick lines* in **B**) and the nuclear ribbons detected on the same section (*arrows*) or on the immediately adjacent sections (*arrowheads*). **C** and **E** show the areas *boxed* in **A** at a higher magnification and viewed with differential interference contrast, together with the corresponding nuclear stains (**D**, **F**). The *arrowheads* in **C**–**F** indicate the position of the ribbons (compare with **B**). *Arabic numerals* mark individual ribbons that extend over almost all lobules, as depicted in Fig. 6. The letter *x* indicates the positions of additional ribbons. *Roman numerals* mark the cerebellar lobules (*EGL* external granule layer, *EthBr* ethidium bromide, *IGL* internal granule layer, *ML* molecular layer, *OLcad* OL-protocadherin). *Bars* **A**, **B** 400 µm; **C–F** 100 µm

previously (Korematsu and Redies 1997a; Hirano et al. 1999), cadherin-8 and OL-protocadherin are each expressed by clusters of immature Purkinje cells (Figs. 1A, arrowheads, 2A). Note that the two cadherins show distinct expression patterns, as previously demonstrated on whole-mount specimens (Suzuki et al. 1997; Hirano et al. 1999). Cadherin-8-positive Purkinje cell clusters are found in all lobules, except lobules I–III. Purkinje cell clusters immunoreactive for OL-protocadherin are also found in all lobules, except lobule I.

In the chicken, granule cell raphes are frequently found at the borders of cadherin-expressing Purkinje cell clusters (Arndt et al. 1998). We therefore also searched for granule cell raphes at the borders of Purkinje cell clusters in the mouse. A nuclear stain of a section through lobules IV–VIII (Fig. 1B) reveals thin ribbons of cell nuclei at the majority of borders of the cadherin-8-positive Purkinje cell clusters (arrows in Fig. 1B). The ribbon pattern is symmetrical about the midline. Additional ribbons bordering the cadherin-8-positive cell clusters were identified on nuclear stains of immediately adjacent sections (arrowheads in Fig. 1C). Similar findings were obtained for the OL-protocadherin-positive Purkinje cell clusters (data not shown). Figures 1C and 2B show schematic superpositions of the cadherin-positive Purkinje cell clusters (thick lines) and the positions of all ribbons of cell nuclei detected in the section shown

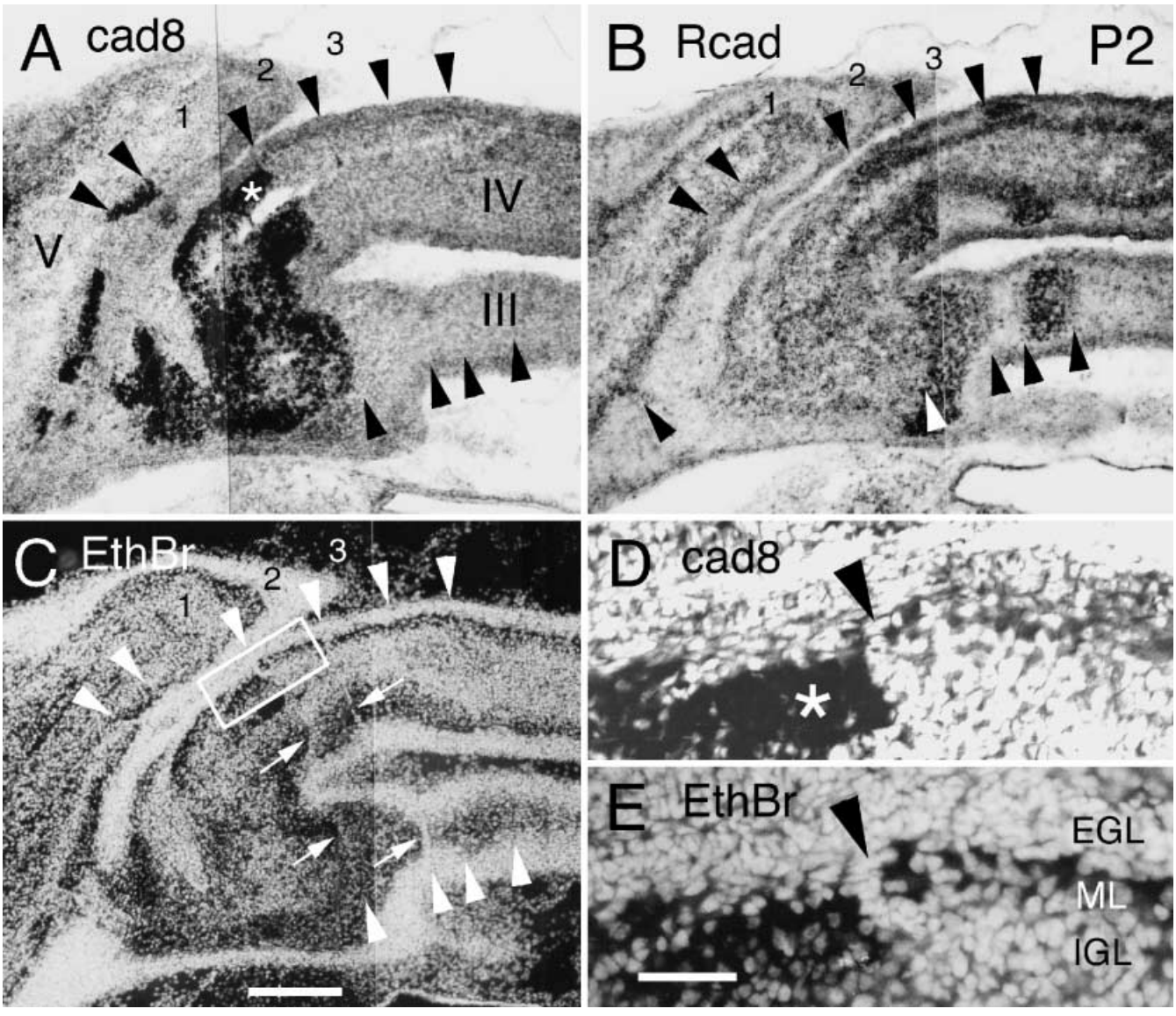

Fig. 4A–E Segments of cadherin-expressing Purkinje cells separated by nuclear ribbons (raphes) in the molecular layer. Transverse sections through P2 cerebellum were hybridized in situ with antisense probes for cadherin-8 (*cad8* in **A**) and R-cadherin (*Rcad* in **B**). The section shown in **A** was counterstained with ethidium bromide for cell nuclei (*EthBr* in **C**). *Arrowheads* mark the borders between the Purkinje cell segments differentially expressing cadherins; some of the segments are numbered (*1–3*). At some of the borders, nuclear ribbons are seen that extend parasagittally across two lobules (*arrows* in **C**). The cadherin-8-positive segment 1 (*asterisk* in **A, D**) is shown at a higher magnification in **D**, together with the corresponding nuclear stain (**E**). The *arrowheads* in **D** and **E** point to a nuclear ribbon in the molecular layer. *Roman numerals* mark the cerebellar lobules (*EGL* external granular layer, *IGL* internal granular layer, *ML* molecular layer). *Bars* **A–C** 200 μm; **D, E** 50 μm

(arrows in Figs. 1C, 2B) and in immediately adjacent sections (arrowheads in Figs. 1C, 2B), confirming the extensive spatial coincidence between the two types of structures in lobules IV-VIII (Fig. 1C) and lobules IV–X (Fig. 2B), respectively. The borders of cadherin-expressing Purkinje cell clusters in the other lobules show a similarly frequent coincidence with the ribbons. Note, however, that a few borders of the cadherin-positive Purkinje cell clusters do not coincide with ribbons of cell nuclei (3 out of the 20 borders shown for cadherin-8 in Fig. 1C, and 7 out of 26 borders shown for OL-protocadherin in Fig. 2B). Vice versa, many nuclear ribbons do not form the borders of cadherin-positive Purkinje cell clusters (17 out of the 33 ribbons shown in Fig. 1C, and 9 out of 28 ribbons shown in Fig. 2B). Similar results were obtained for the other lobules containing clusters of Purkinje cells expressing cadherin-8 or OL-protocadherin.

Figure 3 shows, at a higher magnification, three nuclear ribbons (arrows in Fig. 3C) bordering two cadherin-8-positive Purkinje cell clusters (Fig. 3A) in lobules V and VI of P3 cerebellar cortex. For OL-protocadherin, similar findings are presented in Fig. 2C–F. The ribbons are characterized by a higher density of cell nuclei. Cell nuclei are also found dispersed throughout

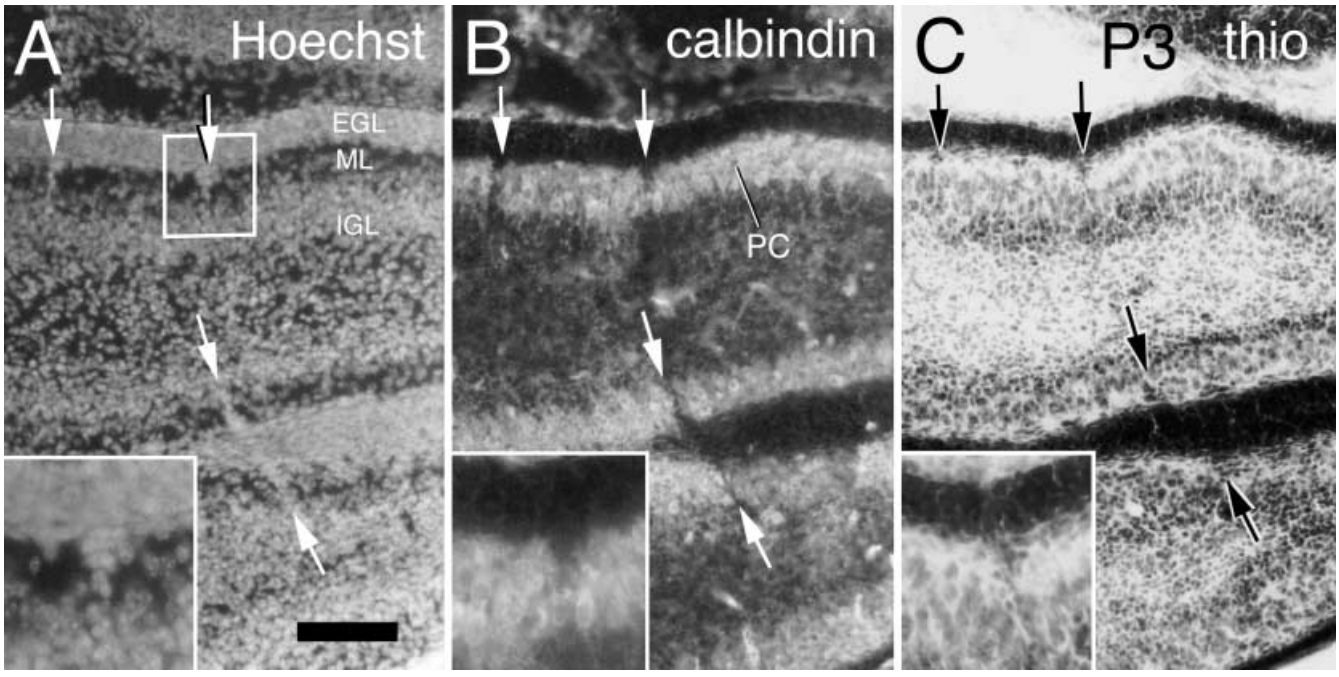

Fig. 5A–C Position of nuclear ribbons (raphes) in the molecular layer in relation to calbindin immunoreactivity and Nissl stains. A transverse section through P3 cerebellum was immunostained with an antibody against calbindin (**B**) and counterstained with nuclear dye Hoechst 33258 (*Hoechst* in **A**). An adjacent section was stained for Nissl substance with thionine (*thio* in **C**). The *arrows* point to the positions of nuclear ribbons in the molecular layer; these positions coincide with gaps in the calbindin immunostain. The *inserts* in each panel show magnifications of the area *boxed* in **A**. *Roman numerals* mark the cerebellar lobules (*IGL* internal granular layer, *EGL* external granular layer, *ML* molecular layer, *PC* Purkinje cells). *Bar* 100 µm

the developing molecular layer but at a much lower density (Figs. 3C, 2D, F).

As in the chicken (Arndt and Redies 1996), some Purkinje cell clusters express R-cadherin. These clusters are partially complementary to the cadherin-8-positive clusters. For example, Fig. 4 shows several segments of Purkinje cells (between arrowheads) that differentially express the two cadherins in lobules III–V of the P2 cerebellum. The segment marked "1" expresses cadherin-8 (asterisks in Fig. 4A, D) but not R-cadherin (Fig. 4B), while the segment marked "2" expresses R-cadherin but not cadherin-8. Neither of the two cadherins is expressed in the segment marked "3". The segment marked "1" is separated from that marked "2" by a nuclear ribbon (Fig. 4E). With few exceptions, nuclear ribbons also border the other cadherin-expressing segments (arrows in Fig. 4C). The cadherin-expressing Purkinje cell segments and the intervening nuclear ribbons can be seen in lobules III, IV, and V at corresponding positions. R-cadherin-positive cells are also found in the external and internal granular layers at P2 (Fig. 4B).

Compared with similar ribbons in the chicken (Arndt et al. 1998; see also Discussion), the ribbons in the mouse are less conspicuous, and it was sometimes difficult to clearly distinguish the ribbons from more loosely dispersed cell nuclei in the molecular layer. Complete series of sections through the entire cerebellar cortex at P3–5 show nuclear ribbons in all cerebellar lobules. Frequently the ribbons can be followed from section to section over several lobules, indicating that they extend parasagittally over large parts of the cerebellar cortex. Given their parasagittal extent, we attempted a complete reconstruction of the ribbon pattern in the cerebellar cortex of the postnatal mouse. For this purpose, the Purkinje cell segments were stained with an antibody against calbindin. As shown previously by Wassef et al. (1985), the Purkinje cells are compartmentalized and there are gaps of calbindin immunoreactivity at regular intervals in the prospective Purkinje cell layer. Double-labeling of a complete series of sections from P3 cerebellum with calbindin antibody and nuclear dye revealed that the gaps (Fig. 5B) are regularly found at a position directly beneath a nuclear ribbon (Fig. 5A). In our experience, the gaps are discernible somewhat more easily and can be followed more consistently from section to section than the ribbons. The positions of the ribbons and/or gaps were mapped over the entire cerebellar cortex in a complete series of sections through P5 cerebellum. The positions were transferred onto anterior and posterior views of the P5 cerebellum, as shown in the schematic diagram displayed in Fig. 6. Results reveal that eight ribbons/gaps extend over almost all cerebellar lobules, and these were numbered in Fig. 6. Additional ribbons/gaps are found only in specific lobules (e.g., see lobules IV–VI in Fig. 6). Consequently, the lobules differ in the number of ribbons/gaps they contain. Generally, the lobules with a larger mediolateral extent contain more ribbons/gaps. Most lobules contain 9–12 ribbons/gaps. Nearly all of the eight ribbons/gaps that extend over the entire cerebellar cortex coincide with borders of

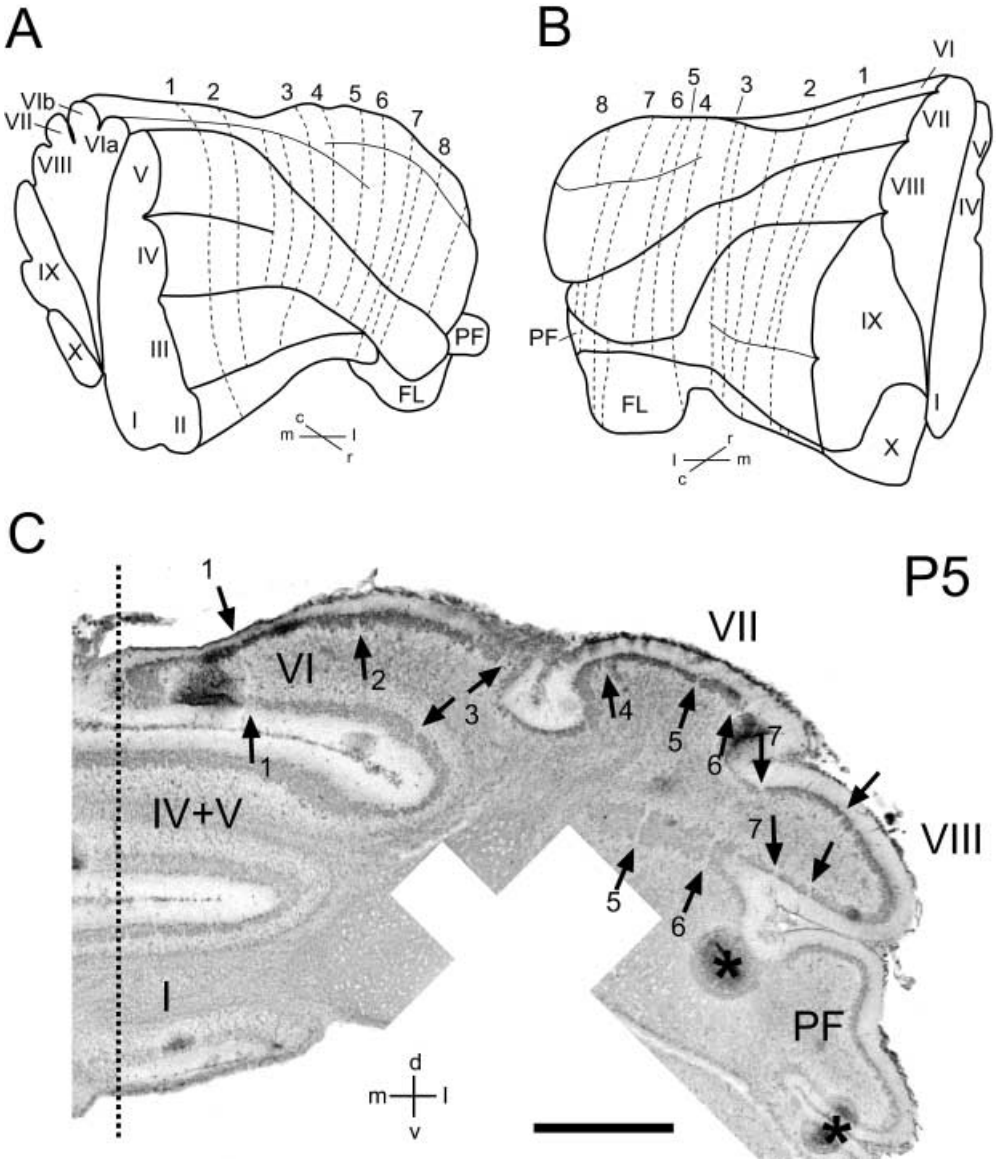

Fig. 6A–C Complete schematic reconstruction of raphes in the P5 cerebellum shown from an anterior-angled view (**A**) and a posterior-angled view (**B**; cerebellar outlines modified after color Figs. 4B and 5B, respectively, by Altman and Bayer, 1997). The analysis was based on a series of transverse sections doubly stained with calbindin antibody and nuclear dye. One representative calbindin-stained section from this series is shown in **C**. The *dashed lines* in **A** and **B** represent the position of the raphes. *Arabic numerals* mark individual raphes which extend from anterior (**A**) to posterior lobules (**B**). *Roman numerals* mark the cerebellar lobules. The *arrows* in **C** point to raphes, as indicated by the gaps in the calbindin staining and/or nuclear ribbons. *Arabic numerals* in **C** refer to individual raphes, as indicated in **A** and **B**. *Asterisks* in **C** mark artifacts. The *dashed line* in **C** represents the midline (*c* caudal, *d* dorsal, *FL* lobulus flocculonodularis, *m* medial, *l* lateral, *PF* paraflocculus, *r* rostral, *v* ventral). *Bar* **C** 500 µm

Purkinje cell cluster that are positive for cadherin-8 or for OL-protocadherin (Figs. 1C, 2B).

Appearance and molecular characteristics of nuclear ribbons in development

At E16 and E18, the molecular layer is relatively thin and no ribbons of increased cell density could be observed. On nuclear stains, ribbons in the molecular layer are first seen around P0. At this stage of development, R-cadherin is expressed by dispersed cells in the nuclear ribbons (arrowheads in Fig. 7B) and in other parts of the molecular layer (arrows in Fig. 7B). Most (if not all) cells in the external and internal granular layers express cadherin-11 (Fig. 7C). Some of the nuclear ribbons are also cadherin-11-positive (arrowheads in Fig. 7C). At P3, most of the cells in the nuclear ribbons express R-cadherin (Fig. 3B). R-cadherin is also strongly expressed in the internal granular layer. In the external granular layer, only weak expression is seen at P3. The ribbons of cells can even be seen on Nissl stains (Fig. 5C). The staining and density of the ribbon cells is similar to the cells in the innermost sheet of the external granular layer (insert in Fig. 5C). We did not observe any TAG-1 expression (Furley et al. 1990; Stottmann and Rivas 1998) in the nuclear ribbons by in situ hybridization or immunostaining. However, the TAG-1-positive external granular layer occasionally formed a shallow bulge into the molecular layer at the position of some of the nuclear ribbons (data not shown). Ribbons are still seen at P6 but, from P7 onwards, they are no longer observed.

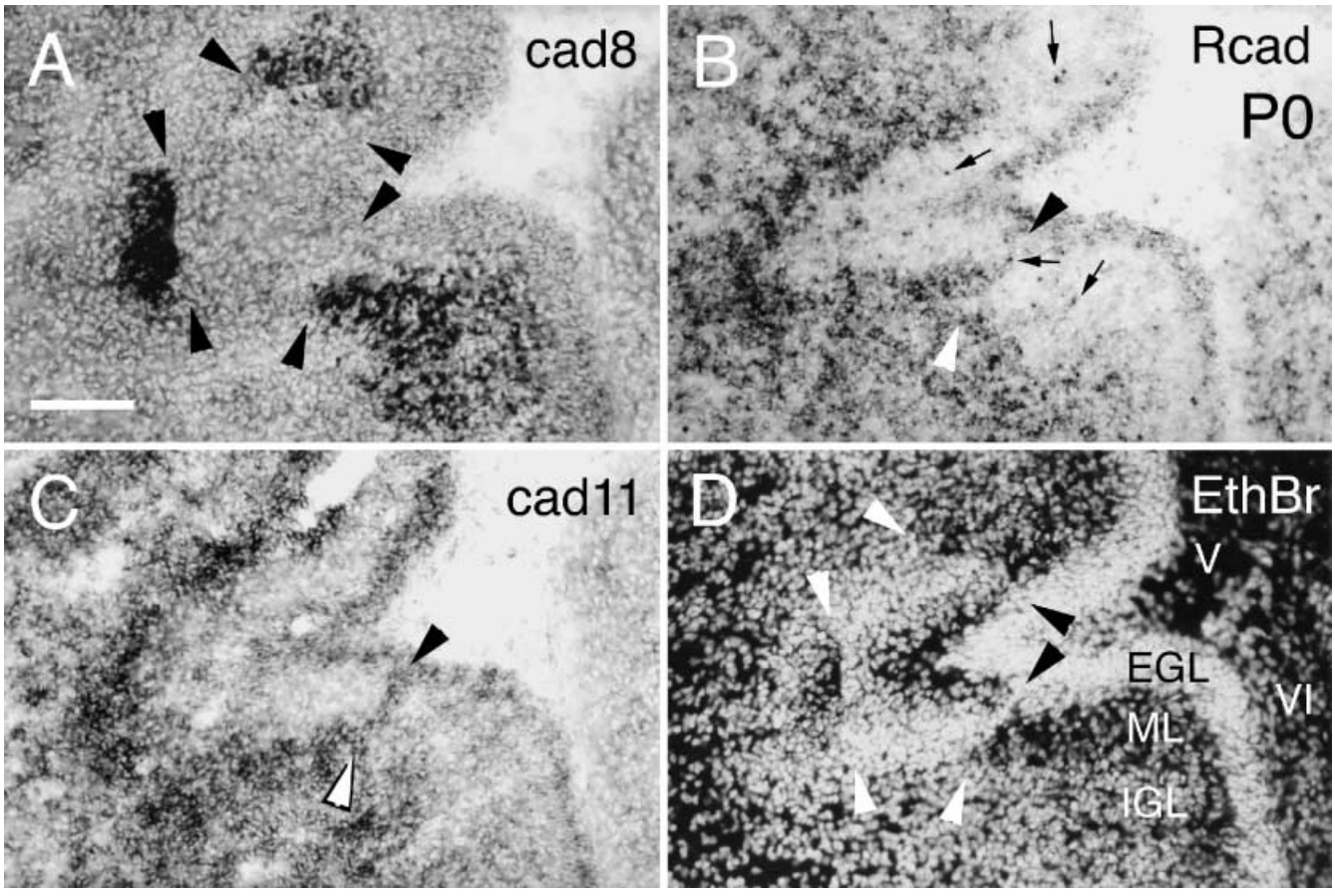

Fig. 7A–D Differential cadherin expression in the cerebellar cortex of P0 cerebellum. Adjacent transverse sections were hybridized in situ with antisense probes for cadherin-8 (*cad8* in **A**), R-cadherin (*Rcad* in **B**) and cadherin-11 (*cad11* in **C**). The section shown in **A** was counterstained with ethidium bromide (*EthBr*) for cell nuclei (**D**). The *arrowheads* point to the borders between cadherin-8-positive Purkinje cell clusters and to nuclear ribbons (raphes) in the molecular layer, respectively. The *arrows* in **B** point to single R-cadherin-expressing cells in the molecular layer. *Roman numerals* in **D** mark the cerebellar lobules (*EGL* external granular layer, *IGL* internal granular layer, *ML* molecular layer). *Bar* 100 μm

Relation of early-onset banding pattern of OL-protocadherin to late-onset banding pattern of zebrin II

OL-protocadherin continues to be expressed by specific Purkinje cell clusters to at least P14. A comparison of the expression patterns at P5, P7, P10, and P14 shows that the Purkinje cell clusters expressing OL-protocadherin at each stage are found at similar topological positions with respect to the evolving pattern of cerebellar foliation and growth in the mediolateral dimension (for review, see Altman and Bayer 1997). Although we could not follow the expression of OL-protocadherin at the level of individual cells, the overall impression is that the Purkinje cell domains that express OL-protocadherin at P5 retain their OL-protocadherin expression at least until P14, irrespective of variations in the level of expression and possible slight expansions or retractions of individual domains. For example, Fig. 8 depicts pairs of OL-protocadherin-immunoreactive Purkinje cell clusters at similar topological positions at P5 and P14 (Fig. 8A and B, D and E, G and H, respectively). At P5, OL-protocadherin immunoreactivity is mostly restricted to the Purkinje cell layer. At P14, the immunoreactivity that extends into the molecular layer probably represents OL-protocadherin-positive dendrites of Purkinje cells. The positional information provided by the expression of OL-protocadherin can thus be related both to the early-onset raphe pattern and to late-onset markers of parasagittal cerebellar banding. In the present study, we compared the expression of OL-protocadherin with that of zebrin II, which is one of the best known late-onset markers (Eisenman and Hawkes 1993; Hawkes and Mascher 1994). This molecule is initially expressed by all Purkinje cells but becomes gradually restricted to subsets of Purkinje cells. In rat, the adult pattern appears from P12 onward (for review, see Hawkes and Mascher 1994). At P14, heterogeneities in the zebrin II staining, including sharp steps in staining, are found in the mouse cerebellar cortex (present results). These heterogeneities have an overall distribution that is highly similar to the pattern of zebrin II-positive and zebrin II-negative compartments in the adult mouse (Eisenman and Hawkes 1993). According to the scheme of zebrin banding in the

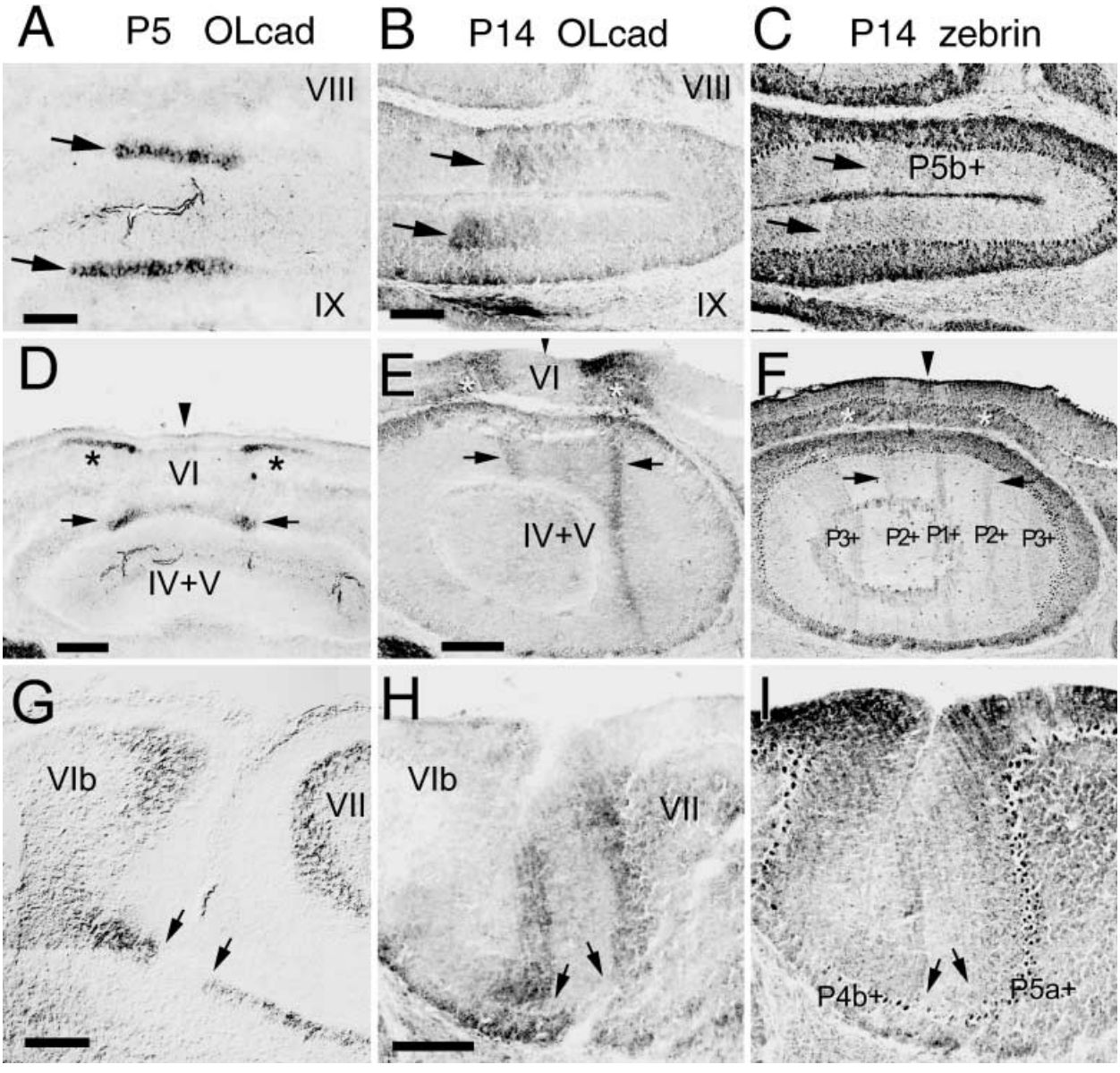

Fig. 8A–I Comparison of immunostaining results for OL-protocadherin at P5 (*OLcad* in **A**, **D**, **G**) and at P14 (**B**, **E**, **H**), and for zebrin II at P14 (*zebrin* in **C**, **F**, **I**). Each *row* shows corresponding regions of developing cerebellar cortex. The results displayed for P14 in **B** and **C**, **E** and **F,** and **H** and **I** are from adjacent sections, respectively. **A** and **G** show enlargements of the areas boxed in Fig. 2A. **D** shows an enlargement of the dorsal midline region of the section displayed in Fig. 2A. The *arrows* mark the positions of borders of immunostaining. The borders marked in **A–C**, **D–F**, and **G–I**, respectively, are located at corresponding positions. The *arrowheads* in **D–F** mark the midline. The *asterisks* in **D–F** indicate the position of two OL-protocadherin-positive Purkinje cell clusters that are symmetrical about the midline. The letters in **C**, **F**, and **I** mark stripes in the zebrin II expression pattern, following the terminology of Eisenman and Hawkes (1993). *Roman numerals* mark the cerebellar lobules. *Bars* **A** 100 µm; **B, C** 200 µm; **D** 200 µm; **E, F** 400 µm; **G** 100 µm; **H, I** 200 µm

adult mouse proposed by these authors, we identified the corresponding bands at P14.

Figure 8 shows that several of the steps in zebrin II immunostaining at P14 (arrows in Fig. 8C, F, I) precisely coincide with borders of OL-protocadherin expression in adjacent sections (arrows in Fig. 8B, E, H). In lobules III–VIII, the OL-protocadherin-positive Purkinje cell clusters overlap extensively with the zebrin-positive bands "P2+," "P4b+," "P5a+," and "P5b+" (terminology according to Eisenman and Hawkes, 1993), whereas the other zebrin-positive bands and all zebrin-negative bands do not express OL-protocadherin. In lobules I, II, IX, and X, the OL-protocadherin immunostaining is less distinct and was difficult to relate to the zebrin II pattern. The arrows in Fig. 8A, D, G indicate borders of OL-protocadherin expression in the P5 cerebellum at corresponding topological positions that, in turn, coincide with ribbons (e.g., compare Fig. 8A with Fig. 2E, F; Fig. 8D with the dorsal midline region in Fig. 2A, B; and

Fig. 8G with Fig. 2C, D). Based on a comparison of several lobules at P14, we identified a correspondence between the topological positions of a ribbon at P5 and a border of zebrin II expression at P14 for the following positions (from medial to lateral): borders of midline band P1+ (Fig. 8F, ribbons marked "x_1" in Fig. 2B), medial and lateral border of band P2+ (Fig. 8F, ribbons marked "1" and "2" in Fig. 2B, respectively), lateral border of band P4b+ (Fig. 8I, ribbon marked "x_2" in Fig. 2B, C, D), medial border of band P5a+ (Fig. 8I, ribbon marked "x_3" in Fig. 2B, C, D), lateral border of band P5b+ (Fig. 8C, ribbon marked "6" in Fig. 2B, E, F).

Discussion

The present study demonstrates parasagittal ribbons of cells in the molecular layer of mouse cerebellar cortex during the 1st postnatal week of development. These ribbons extend from the external granular layer into the prospective Purkinje cell layer. The ribbons are found in nearly all cerebellar lobules (Fig. 6). In the following paragraphs, we will discuss the evidence that the ribbons correspond to the granule cell raphes first described by Feirabend (1983, 1990) in the chicken, followed by a discussion of the distribution of the granule cell raphes in the mouse cerebellar cortex and their relation to early-onset and late-onset parasagittal banding patterns of cerebellar cortex.

Evidence for granule cell raphes in the mouse cerebellar cortex

The following results strongly suggest that the cell ribbons found in the postnatal mouse cerebellum in the present study are the mouse equivalent of the granule cell raphes present in chicken:

1. Both in chicken (Arndt et al. 1998; Lin and Cepko 1998) and in mouse, the ribbons are located at the borders of distinct Purkinje cell segments, as revealed in the present study by their expression of cadherin-8, OL-protocadherin and R-cadherin (Figs. 1, 2, 3, 4, 7, 8; Korematsu and Redies 1997a; Suzuki et al. 1997; Hirano et al. 1999). Such a relationship was found in lobules III–VI (as shown in Figs. 1, 4, 7, 8) and in most of the other lobules. Molecularly distinct Purkinje cell segments have been demonstrated previously at similar stages of mouse cerebellar development with other molecular markers (e.g., GMP-dependent protein kinase, vitamin D-dependent calcium-binding protein; Wassef et al. 1985; see reviews by Herrup and Kuemerle 1997; Oberdick et al. 1998). In the chicken, other cadherins (cadherin-6B and cadherin-7, Arndt et al. 1998; and cadherin-10, Fushimi et al. 1997) are also expressed by distinct parasagittal Purkinje cell clusters. It is at present unclear whether the expression domains of additional cadherins expressed in the mouse cerebellum (see Suzuki et al. 1997) are similarly delimited by ribbons. It is conceivable (but highly speculative) that most (if not all) ribbons will eventually be found to form boundaries of cadherin-expressing Purkinje cell clusters in both species. In the chicken, some raphes were also found inside Purkinje cell domains expressing cadherin-6B and cadherin-7 (Fig. 3A–C by Arndt et al., 1998). In the present study, raphes inside cadherin-positive Purkinje cell domains were not observed.
2. In both chicken and mouse, the ribbons extend parasagittally over several lobules or, in some cases, over the entire cerebellar cortex. The number and spacing of the ribbons is roughly similar in mouse and in chicken (compare our Fig. 6A, B with Fig. 59 by Feirabend, 1990).
3. In the mouse, most cells in the ribbons have a nuclear size and histological appearance in thionine stains similar to the granule cells of the innermost sheet of the external granular layer (Fig. 5C). The ribbon cells can be followed into the layer of prospective Purkinje cells (insert in Fig. 5C). Similar observations were made in the chicken (Feirabend 1983, 1990).
4. As in the chicken, the majority of ribbon cells express specific cadherins (e.g., cadherin-11 in mouse; see Fig. 7C; cadherin-7 and –10 in chicken; Fushimi et al. 1997; Arndt et al. 1998). The same cadherins are also expressed by the granule cells in the differentiating internal granular layer (Fig. 7C; Arndt et al. 1998). In chicken, R-cadherin is expressed only by a small subpopulation of ribbon cells that probably represent migrating interneurons (Arndt et al. 1998). Scattered R-cadherin-expressing cells are also found in the ribbons of mouse at stage P0, as well as dispersed throughout the molecular layer and the internal granular layer (Fig. 7B). Whether these cells also represent interneurons remains to be demonstrated. However, at P3, R-cadherin-expressing cells are more numerous in the ribbons and the granular layers, suggesting that at least some granule cells begin to express R-cadherin around this stage of development (Fig. 3B).
5. In both chicken and mouse, the ribbons are found at similar stages of cerebellar development (P0–6 in mouse compared with E9–15 in chicken). At later stages, granule cell migration through the molecular layer is more widespread and granule cell raphes are no longer observed.
6. The ribbon cells do not express TAG-1, a marker for cells in the innermost external granular layer (data not shown; Yamamoto et al. 1986; Stottmann and Rivas 1998). Similar results were obtained in the chicken (K. Arndt and C. Redies, unpublished observations). TAG-1 expression was also reported to be absent in granule cells migrating through the molecular layer (Stottmann and Rivas 1998).

In conclusion, we propose that the ribbons found in the mouse in the present study correspond to the granule cell raphes described by Feirabend (1983) for the chicken.

We therefore also refer to them as *granule cell raphes* in the mouse. Lin and Cepko (1998) have previously searched for granule cell raphes in mouse but have failed to detect them. Indeed, the mouse raphes are not as prominent as those in the chicken, and it was sometimes difficult to consistently follow an individual raphe from section to section. For this reason, we analyzed several adjacent sections before determining whether or not a raphe was present at a particular cortical cerebellar location.

Note that the cell-dense granule cell raphes in the molecular layer described in the present study are distinctly different from the cell-poor (medullary) raphes described by Korneliussen (1967, 1968). The latter type of raphe is found between clusters of prospective Purkinje cells and between the deep cerebellar nuclei at earlier embryonic stages of mammalian cerebellar development than the granule cell raphes investigated in the present study. Whether the two types of "raphe" relate to each other is unclear.

The cells in the granule cell raphes do not express calbindin, a marker for Purkinje cells (Fig. 5B; Wassef et al. 1985; Iacopino et al. 1990). At the position of the raphes, gaps in calbindin staining are observed in the prospective Purkinje cell layer (Fig. 5B). Such gaps have been noticed before and were postulated to separate adhesive Purkinje cell compartments (Wassef et al. 1985). They were interpreted as being similar to the cell-poor (medullary) raphes of Korneliussen (1967, 1968; Wassef et al. 1985). Note, however, that the gaps in the layer of prospective Purkinje cells are densely packed with cells. At least some of these cells have a histological appearance similar to the cells in the granule cell raphes (Fig. 5C). Consequently, we propose that the gaps contain an extension of the raphes (see also Lin and Cepko 1998). The possibility that the gaps in calbindin immunostaining also contain calbindin-negative Purkinje cells cannot be excluded.

Overall organization of the pattern of granule cell raphes

In the chicken, step-like changes in the expression of a large variety of different early-onset markers have been shown to coincide with raphes (Arndt et al. 1998; Lin and Cepko 1998). The raphe pattern in the chicken, and possibly also that in the mouse, can thus be interpreted as a positional reference system that represents a common topological framework underlying the patterns of many early-onset markers of parasagittal banding. Given the likely importance of the raphe pattern as an indicator of mediolateral cerebellar compartmentation, we mapped the raphe pattern over the entire cerebellar cortical surface of the mouse at P5. The combination of raphes in the molecular layer and their extension into the gaps of calbindin immunostaining in the Purkinje layer allowed us to map the position of the raphes (gaps) in a consistent way. Most raphes extend along several lobules in the anteroposterior dimension (Fig. 6A, B). Only about 6–8 raphes extend over almost the entire cortical surface. This number is similar to the number of mediolateral compartments that were previously postulated on the basis of mapping common expression boundaries for multiple parasagittal markers (Herrup and Kuemerle 1997; Oberdick et al. 1998). The precise number of the compartments in each lobule, however, is a matter of uncertainty and scientific debate, given the large number of domains that could potentially be defined by combining the partially overlapping expression patterns of multiple markers. Indeed, we found additional raphes that are likely to border additional compartments in several lobules (Fig. 6). This result is consistent with the finding that the number of parasagittal stripes or patches, e.g., of cadherin expression, varies between the lobules (see Suzuki et al. 1997; Hirano et al. 1999). Moreover, in the chicken, bifurcations of individual raphes have been observed (Feirabend 1990). In our study, we could not observe such bifurcations, but this failure may be due to the fact that the mouse raphes are less prominent than those in the chicken. Nevertheless, it is noteworthy that the overall number of raphes found here in the mouse at P5 approximately matches that of chicken at E14 (compare our Fig. 6A, B to Fig. 59 by Feirabend, 1990). Another marker of parasagittal banding, zebrin II, has also been reported to show an evolutionarily conserved distribution in different species (for review, see Herrup and Kuemerle 1997).

Relating positional cues from early-onset markers to those of late-onset markers of parasagittal compartmentation

One of the unsolved problems of cerebellar development is how positional cues in the early-onset pattern of parasagittal banding relate to those in the late-onset pattern. The present study demonstrates that the expression of OL-protocadherin is topologically relatively invariant from P5 to at least P14, despite the considerable growth of the cerebellum during this period. Although not demonstrated at the single-cell level, individual OL-protocadherin-positive Purkinje cell clusters can be followed as distinct entities that retain their approximate position at least in some cerebellar lobules (lobules IV–IX; Fig. 8). In these lobules, the borders of OL-protocadherin expression often coincide with the position of raphes at P5. At P14, the borders of OL-protocadherin-positive Purkinje cell clusters at the same topological position, in turn, regularly coincide with heterogeneities in the expression pattern of zebrin II, a late-onset marker of parasagittal banding (Fig. 8). This result provides indirect evidence for a spatial relationship between the early-onset banding pattern (as represented, e.g., by the raphes) and late-onset banding patterns. Specifically, the results indicate that at least some of the positional cues carried by the compartmental boundaries are shared by the two types of patterns, as previously postulated by other researchers based on other evidence (see Herrup

and Kuemerle 1997). Whether or not exactly the same population of Purkinje cells carries these cues remains unclear, because we could not monitor individual Purkinje cells and their expression of OL-protocadherin in the vicinity of the boundaries during development. It should be noted that not all positional cues must be shared between the two types of patterns. Baader et al. (1999) recently reported that the ectopic expression of the transcription factor engrailed-2 in cerebellar Purkinje cells in mutant mice selectively disrupts late-onset patterns but not early-onset patterns. Two possible interpretations for this finding were proposed: (1) two (at least partially) distinct mediolateral boundary systems exist early and late in cerebellar development; (2) there is only one boundary system, which fails to be translated from early to late stages of development in the mutant mice (Baader et al. 1999).

Open questions on the role of the raphes in functional cerebellar patterning

The present work is descriptive and no experimental evidence for a functional role of the raphes in cerebellar patterning is provided. The following hypotheses are based on the timing of appearance of the raphes and remain speculative. When granule cell raphes first appear in the molecular layer (around P0), the parasagittal patterning and segmentation of prospective Purkinje cells is already well advanced. Preceding the appearance of the raphes, the prospective Purkinje cell layer shows parasagittal expression of several gene regulatory proteins (see Millen et al. 1995) and of cadherins (Arndt and Redies 1996; Korematsu and Redies 1997a,; Arndt and Redies 1998). The raphe pattern is therefore likely to be a consequence of primary cortical cerebellar patterning rather than its cause. At the time when the raphes appear in the postnatal cerebellum, each segment of Purkinje cells contains several layers of cadherin-expressing cells. It is conceivable that these segments are tightly adherent due to their cadherin expression and that they constitute a barrier for migrating granule cells. In this scenario, the border regions between the segments would represent a route of migration that is more permissive for granule cell migration that the Purkinje cell segments themselves. Once the granule cell raphes have formed, they might serve to separate the Purkinje cell clusters, thus stabilizing mediolateral boundaries in the developing cerebellar cortex. A similar function of cerebellar granule cells has recently been demonstrated experimentally in Unc5h3 mutant chimeric mice with cerebellar ectopias. Here, the granule cell was interpreted to be the pioneer cell type to demarcate boundaries inside and outside the cerebellum (Goldowitz et al. 2000).

Whether the patterned migration of granule cells has an effect on the extension of parallel fibers into the molecular layer remains to be studied. Similarly open is the question of whether the transient concentration of migrating granule cells to the raphes affects intracortical connectivity patterns. Also, the precise relation of the raphe pattern to the parasagittal organization of afferent and efferent connectivity of the vertebrate cerebellum (for review, see Herrup and Kuemerle 1997; Voogd and Glickstein 1998) is unclear at present. A relation between cerebellar fiber connections and the parasagittal expression of adhesion molecules has been demonstrated for several adhesion molecules such as BEN/SC1/DM-GRASP and some cadherins (Chédotal et al. 1996; Arndt and Redies 1998; Arndt et al. 1998). Although some of the common boundaries in the parasagittal banding patterns can be related to innervation patterns (Gravel et al. 1987; Gravel and Hawkes 1990; Hawkes and Mascher 1994), experimental evidence indicates that mediolateral cerebellar patterning takes place also in the absence of afferent fiber input, based on genetic mechanisms that are partially intrinsic to cerebellar tissue (Wassef et al. 1990; Oberdick et al. 1993; Chédotal et al. 1996).

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