Anat Embryol (1994) 190:417–428

Anatomy and Embryology

Original articles

Purkinje cell compartments in the reeler mutant mouse as revealed by Zebrin II and 90-acetylated glycolipid antigen expression

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Accepted: 18 April 1994

Abstract. The cerebellum is organized into a series of parasagittally aligned bands that may be revealed histologically in the adult mouse by largely complementary immunostaining of Purkinje cell sets with the monoclonal antibodies Zebrin II (ZII; antigen:aldolase C) and P-path (PP; antigen:90-acetyl glycolipids). We compared the normal staining pattern using these markers and an antibody to calbindin with that found in the reeler mutants (*rl/rl*), in which most Purkinje cell migration is halted beneath the cerebellar white matter. The results revealed that Purkinje cells in reeler mutants, despite their ectopic location in large subcortical masses, show a clear tendency to distribute into alternating zones that either stain for Zebrin II or for P-path, with variable transition zones of mixed labeling. However, the estimated number of zones was fewer than in the normal adult cortex: roughly 7–9 zones are revealed per side in the mutant compared with 14 major divisions in wild type mice. These results raise the possibility that neurons destined to express these markers are segregated during their migration and that the final phase of migration into the cortex might involve further splitting or interdigitation between cell sets expressing the two antigens.

Key words: Cerebellum – Neurological mutant – Ganglioside – Immunohistochemistry – Neuron migration

Introduction

The mammalian cerebellar cortex exhibits patterns of afferent and efferent connectivity that conform to longitudinal zones (Voogd 1969; Voogd et al. 1985; Buisseret-Delmas and Angaut 1993). A similar parasagittal zonal organization is revealed by histochemical staining for certain metabolic enzymes, such as acetylcholinesterase, 5′-nucleotidase, and cytochrome oxidase (Scott 1964; Voogd 1969; Boegman et al. 1988; Eisenman and Hawkes 1989; Leclerc et al. 1990), and, even more clearly, by immunocytochemical staining with antibodies to the Zebrin I and II antigens present in Purkinje cells (Hawkes et al. 1985; Hawkes and Leclerc 1987; Brochu et al. 1990; Eisenman and Hawkes 1993). The antigenic compartmentation of Purkinje cells is spatially correlated with projection patterns of olivary climbing fibers and with that of some mossy fiber and corticonuclear projections (Hawkes and Leclerc 1986; Gravel et al. 1987; Gravel and Hawkes 1990; Matsushita et al. 1991). Thus, Zebrin immunostaining provides a powerful method for investigation of the effects of experimental or genetic perturbations on the development of a fundamental aspect of cerebellar organization (Wassef et al. 1987; Leclerc et al. 1988; Rouse and Sotelo 1990; Sotelo and Wassef 1991; Tano et al. 1992; Edwards et al. 1994).

The parasagittal zonation of the cerebellum poses a major challenge for developmental biology: does cell fate with respect to antigenic compartments arise from determinative events during histogenesis and cell migration or through cellular interactions with their environment after the Purkinje cells are assembled in the cortex? In the homozygous reeler mutant mouse, most Purkinje cells abort their migration and are assembled in large subcortical masses beneath the white matter (Goffinet 1983; Yuasa et al. 1993). For this reason, study of the reeler mutant allows one to assess which aspects of cerebellar compartmental organization are or are not dependent on the completion of Purkinje cell migration to their normal cortical position.

The reeler mutant phenotype involves gross disturbances in cell position in the cerebral cortex, hippocampus, and cerebellum and more subtle effects on cellular organization in other structures (reviews: Caviness et al. 1988; Goffinet 1984). In the cerebral cortex, the stratification of neuronal classes into layers is largely inverted and

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dendritic orientation is often abnormal, yet connectivity patterns are normal with respect to target cells and topography. The systematic disturbance of cell position arises from a failure of successive waves of neurons generated in the ventricular zone to migrate past their predecessors, a sequence that normally leads to an inside-out pattern of cell position with respect to cell birthdate. In the reeler mutant cerebellum, most Purkinje cells are concentrated in ectopic subcortical masses, which are well segregated from the deep nuclei, and smaller numbers are located in the granule cell layer and in the Purkinje cell layer, which together form an unfoliated cortex with an abnormally small surface area (Meier and Hoag 1962; Mariani et al. 1977; Goffinet et al. 1984; Heckroth et al. 1989; Inoue et al. 1990). Whereas the neurons of the mutant cerebellar cortex show a virtually normal organization with respect to lamination, cell morphology and connectivity at the ultrastructural level, the subcortical Purkinje cells exhibit stunted and maloriented dendritic trees that receive an appropriate innervation from climbing fibers, no parallel fiber input, and an abnormal direct innervation from mossy fibers (Mariani et al. 1977; Wilson et al. 1981; Goffinet et al. 1984; Blatt and Eisenman 1988). In contrast to the neocortex, neuron numbers in the mutant cerebellum are substantially reduced: Purkinje cell number is half that found in normal mice (Blatt and Eisenman 1988), and, judging from the small cortical surface area, the population of granule cells and other interneurons is even more affected. It has been proposed that in both the neocortex and cerebellum the phenotypic abnormalities of the mutation arise as a consequence of a primary disturbance in the interactions between migrating neurons and the radial glial fibers they travel along (Pinto-Lord et al. 1982; Yuasa et al. 1993). However, the affected gene on chromosome 5 has not been identified, and, consequently, the primary site (or sites) of action of the two known mutant alleles is still uncertain.

Some results of previous studies on the reeler cerebellum are relevant to the issue of zonal organization within the subcortical Purkinje cell masses. For example, anterograde labeling of parts of the olivocerebellar system produces longitudinal banding patterns (Goffinet et al. 1984; Blatt and Eisenman 1988), and discontinuities in 5′-nucleotidase staining have been noted (Eisenman 1988). More significantly, Sotelo and Wassef (1991) have reported in abstract form that the subcortical Purkinje masses exhibit segregated zones of Zebrin I immunoreactivity. The present study seeks to provide more direct and detailed information about the organization of the Purkinje cell compartments in the reeler mutant through the use of two selective markers of Purkinje cell subsets, the Zebrin II antibody and the recently developed antibody, P-path. The latter has been shown to recognize 90-acetylated glycolipids and to stain predominantly the Zebrin II-negative Purkinje cell bands (Leclerc et al. 1992). The results on the reeler mutant support the hypothesis that compartmental cell fate in the cerebellum is determined by fundamental events of differentiation early in embryogenesis.

Materials and methods

Animals

Adult mice (older than P50) and 4-week old mice of the hybrid strain B6C3 (C3H X C57BL/6J), which bears the *reeler* mutation (*rl*), were bred in the Shriver Center colony.

Antibodies

The Zebrin II monoclonal antibody (IgG class) was generated in mouse using cerebellar homogenates from the fish *Apteronus* as the immunogen (Brochu et al. 1990). It is known to recognize a 36-kDa protein in Purkinje cells identified as a variant of the cytoplasmic enzyme aldolase C (Hawkes 1992). The P-path monoclonal antibody (IgM class) was generated using fetal rat brain homogenate as the immunogen and has been shown to recognize a set of four glycolipids including 90-acetyl GD3 (Leclerc et al. 1992) and probably 90-acetyl disialoparagloboside (LD1; Chou et al. 1990). The antibody to calbindin (gift of Charles Gerfen, NIMH) is a rabbit polyclonal antibody raised against purified calbindin (vitamin D-dependent 28-kDa protein).

Immunohistochemistry

After anesthetization with ether or Avertin (11 mg/kg), the mice were perfused intracardially with saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.3). The brains were postfixed 6–15 h. at 4°C, immersed in 25% sucrose PB for 4 to 15 h., and sectioned transversely through the cerebellum at 40 mm with a sledge microtome.

Serial sections were collected in 0.05 M phosphate-buffered saline at pH 7.4 (PBS), washed (3 × 15 min) and incubated 15–18 h at 4°C in Zebrin II hybridoma supernatant (1:5–30), P-path hybridoma supernatant (1:2–10), and/or anti-calbindin antibody (1:700–1000) diluted in PBS with 2–10% normal goat serum (Gibco) (NGS-PBS). Sections were washed with PBS, incubated with HRP-conjugated goat anti-mouse IgG or IgM or with goat anti-rabbit IgG for 60–90 min. (Boehringer-Mannheim; 1:50 in NGS-PBS), washed with PBS or 0.05 M TRIS-buffered saline (TBS, pH 7.4), and reacted with TRIS-buffered 0.05% diaminobenzidine (DAB) and 0.005% H_2O_2. Sometimes nickel ammonium sulfate was added to the DAB solution (2 mg/ml) in order to darken the reaction product. Control sections were processed without a primary antibody.

For double-immunostaining, both hybridoma supernatants were applied simultaneously, and successive incubations were done with each secondary antibody and two different DAB solutions, one containing nickel ammonium sulfate, which yields a black reaction product, and the other without heavy metals, yielding a brown reaction product (Hancock 1986). The order of the latter steps was alternated between cases. In addition, some sections from reeler and wild-type cases were processed for double-immunofluorescence using an FITC-tagged secondary antibody to reveal P-path immunoreactivity (Boehringer-Mannheim) and a biotinylated secondary antibody followed by a rhodamine conjugated avidin derivative to reveal Zebrin II immunoreactivity (ExtrAvidin-RITC, Sigma).

Analysis

In the reeler mutant, photographic analysis was supplemented by a 3D reconstruction of a case processed for double- labeling in serial sections. Through use of a camera lucida attached to a Leitz Orthoplan microscope, charts of all 30 sections through the cerebellum were made at low magnification (× 45). These charts contained color-coded outlines of zones containing one of three types of stain-

ing based on cells and surrounding neuropil: Zebrin only, P-path only, or mixed. The latter category included areas with individual cells that were double-labeled and/or areas where neuropil and cells of both staining types were highly intermixed. Photocopied transparencies of 16 closely spaced drawings were aligned and digitized with a Summagraphics tablet interfaced to an IBM compatible personal computer running DesignCAD 3D (American Small Business Computers). Relative section position was encoded in Z axis coordinates. Using a special command to create a virtual surface over selected lines, a wire-frame mesh of 20–40 planes was constructed around profiles of like labeling that lined up in sets of 3–4 sections visualized at a time. In cases of a divergence of one profile into two, or convergence of two into one, the single profile was segmented into two pieces. A shading subroutine was used to render 3D views of the surfaced labeling zones from selected perspectives at a resolution of 800 × 600 pixels and 8 bits of color information. These views were stored as bitmap files in PCX format and imaged onto color slides by a computer graphic slide bureau (Visual Horizons, Rochester, N.Y.).

In order to assess the relative representation of Zebrin II- and P-path-positive cells in the subcortical Purkinje masses, the relative volumes of the three types of zones were determined by the point-counting stereological method (Underwood 1970). The charts of 16 evenly spaced sections were laid over a rectangular grid of points spaced at 4-mm intervals, and the number of intercepts of each type of Purkinje cell zone was tabulated. Data were combined for four sections in each quartile in the rostrocaudal dimension.

Results

Normal mice

Immunocytochemical results in wild type and phenotypically normal reeler heterozygotes (+/*rl*) reveal that the Zebrin II and P-path monoclonal antibodies stain longitudinal bands of Purkinje cells that are largely complementary (Fig. 1). The results confirm those obtained previously in the C57BL/6 strain (Leclerc et al. 1992). The Zebrin-positive bands tend to be thinner in rostral lobules and thicker in caudal lobules, whereas the converse is true for most P-path-positive bands. The bilaterally symmetrical Zebrin-positive zones include three bands in the vermis (P1-P3), one band that spans the vermis-hemisphere junction (P4), three major bands in the hemisphere (P5-P7), and most of the flocculus and paraflocculus (Fig. 1 b, d, f) (cf also Tano et al. 1992, and Eisenman and Hawkes, 1993). P-path stains all Zebrin-negative zones and, in addition, the P3 Zebrin-positive band throughout its rostrocaudal extent, although with less intensity (Fig. 1 a, c, e). In certain lobules, overlapping expression of Zebrin II and P-path antigens is detectable in the P2 (dorsal regions of lobule IX) and P4 bands (lobule V and ventral lobule VIII). Zones of dual staining are also evident in the hemispheres, the flocculus and paraflocculus.

Reeler mutant mice

In 4-week-old reeler mutants stained with the calbindin antibody (Fig. 2a), the subcortical masses of Purkinje cells show a consistent organization into lateral (L), intermediate (I) and medial (M) subdivisions, as described previously (Goffinet et al. 1984). A small proportion of cells, estimated to comprise about 5% of the Purkinje population (Heckroth et al. 1989), are normally positioned in a thin cortex with an abnormally small surface area and minimal signs of foliation. In the subcortical masses, most of the medial and intermediate subdivisions are immunoreactive for P-path (Fig. 2b); however, calbindin immunostaining reveals that distinct regions of these masses and the lateral perimeter of the lateral Purkinje mass are P-path-negative (compare arrowheads in Fig. 2a, b). Staining with Zebrin II is very weak in the mutant at this age (not shown). Nevertheless, the presence of Purkinje cell zones that are P-path-negative indicates a clear biochemical heterogeneity within the ectopic Purkinje cell population.

In the adult reeler mouse, the subcortical masses were found to be comprised of segregated zones containing P-path positive cells or Zebrin II positive cells. A third type of compartment, also found in the normal cerebellar cortex, shows staining with both antibodies. The overall patterns of labeling are illustrated by photomicrographs from a case with alternate staining of serial sections with the two antibodies (Fig. 2c–g). Subsequent to this description, we will render in more detail aspects of the spatial configuration of the subcortical zones with single and dual labeling as revealed by analysis and a 3D reconstruction based on sections processed for combined labeling with both antibodies (Figs. 3, 4). Finally, observations on antigen staining patterns within the mutant cortex will be presented (Fig. 5).

In transverse sections through a central level with respect to the rostrocaudal extent of the cerebellum, the zones with P-path staining are more extensive in the medial and intermediate cell masses (Fig. 2c). In the medial cell mass, a large dome-like zone of P-path immunostaining is flanked laterally by vertical strips containing Zebrin II reactive cells (Fig. 2d, e, open arrows). Also, a thin column of Zebrin II positive cells lies on each side of a midline septum (Fig. 2d, e, small arrows), which is devoid of Purkinje cells (note the calbindin negative septum in Fig. 2a). These vertically oriented zones of Zebrin II staining become wider in more caudal sections through the cerebellum (Fig. 2g). At this level, it becomes clear that the peri-midline zone contains label with both antibodies (Fig. 2f, g, small arrows), whereas the lateral zone of Zebrin II staining (Fig. 2g, open arrows) only partially overlaps the P-path-stained zone, which contains subzones of intense and weak P-path staining (Fig. 2f, arrowheads). In the intermediate cell mass, (Fig. 2c, d, f, g, diamonds), the extensive P-path-stained region is bounded dorsomedially by strips of Zebrin II (Fig. 2d, g, arrowheads). These caps of Zebrin II staining are in continuity with a stream of reactive cells that extend ventrally across the P-path staining region (Fig. 2g, arrowheads). Finally, in the lateral cell mass, a large vertically elongated zone stained with Zebrin II, which includes ventral, lateral, and dorsal concentrations of stained cells (Fig. 2d, g, arrowheads), may be seen to enfold a Zebrin II negative Purkinje cell zone that is positive for P-path (Fig. 2c, d, f, g, triangles). At the more rostral level, the ventrolateral enlargement of the lateral Purkinje cell mass, which is considered to be homologous to the floccular/paraflocular lobe in normal mice (Goffinet et al,

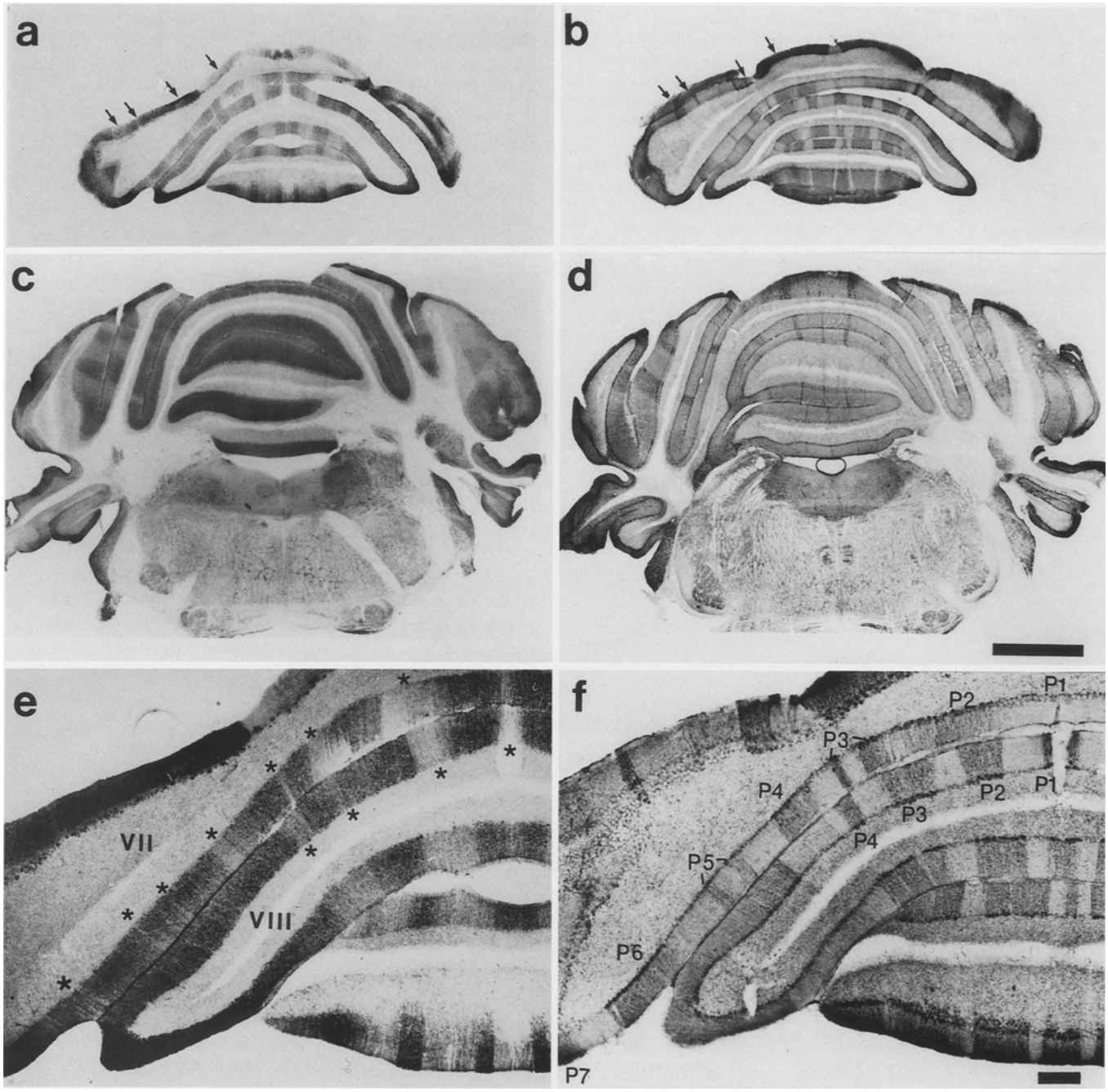

Fig. 1a–f. Pattern of Zebrin II and P-path immunostaining in the wild-type B6C3 mouse. Transverse sections through the cerebellum at caudal (**a, b**) and rostral (**c, d**) levels. Zebrin II antibody staining (ZII; **b, d, f**) and P-path-antibody staining (*PP*; **a, c, e**) show a largely complementary pattern. *Arrows* (**a, b**) point to some of the Zebrin II positive bands that are P-path negative. Note that the width of Zebrin II-reactive bands in the rostral vermis (**d**) is less than that in the caudal section (**b**). In converse fashion, P-path-reactive bands widen as one passes from caudal (**a**) to rostral (**c**) in the vermis, to the point that P-path-negative zones are not discernible at this low magnification. Panels **e** and **f**, which are enlarged views of lobules VII and VIII from **a** and **b**, show the alignment of P-path-negative or -positive bands, marked with *asterisks* in **e**, and Zebrin II-positive bands, labeled *P1–P7* in **f**. An obvious Purkinje cell zone that stains for both markers is the $P3^+$ band. *Bars*: equal 1 mm in **a–d** and 100 mm in **e–f**

1984), is essentially devoid of P-path staining (Fig. 2c, asterisk).

Double-labeled material was used to identify the ZII-positive and P-path-positive overlapping zones. The boundaries between zones of ZII staining and P-path staining were usually found to involve transition zones of variable width that showed staining with both antibodies (Fig. 3). Double-labeled zones in the mutant subcortical masses usually include a mixture of single-labeled cell bodies and their dendritic processes around the cell bodies. Since it was often difficult to discern whether the brown DAB reaction product associated with Zebrin II

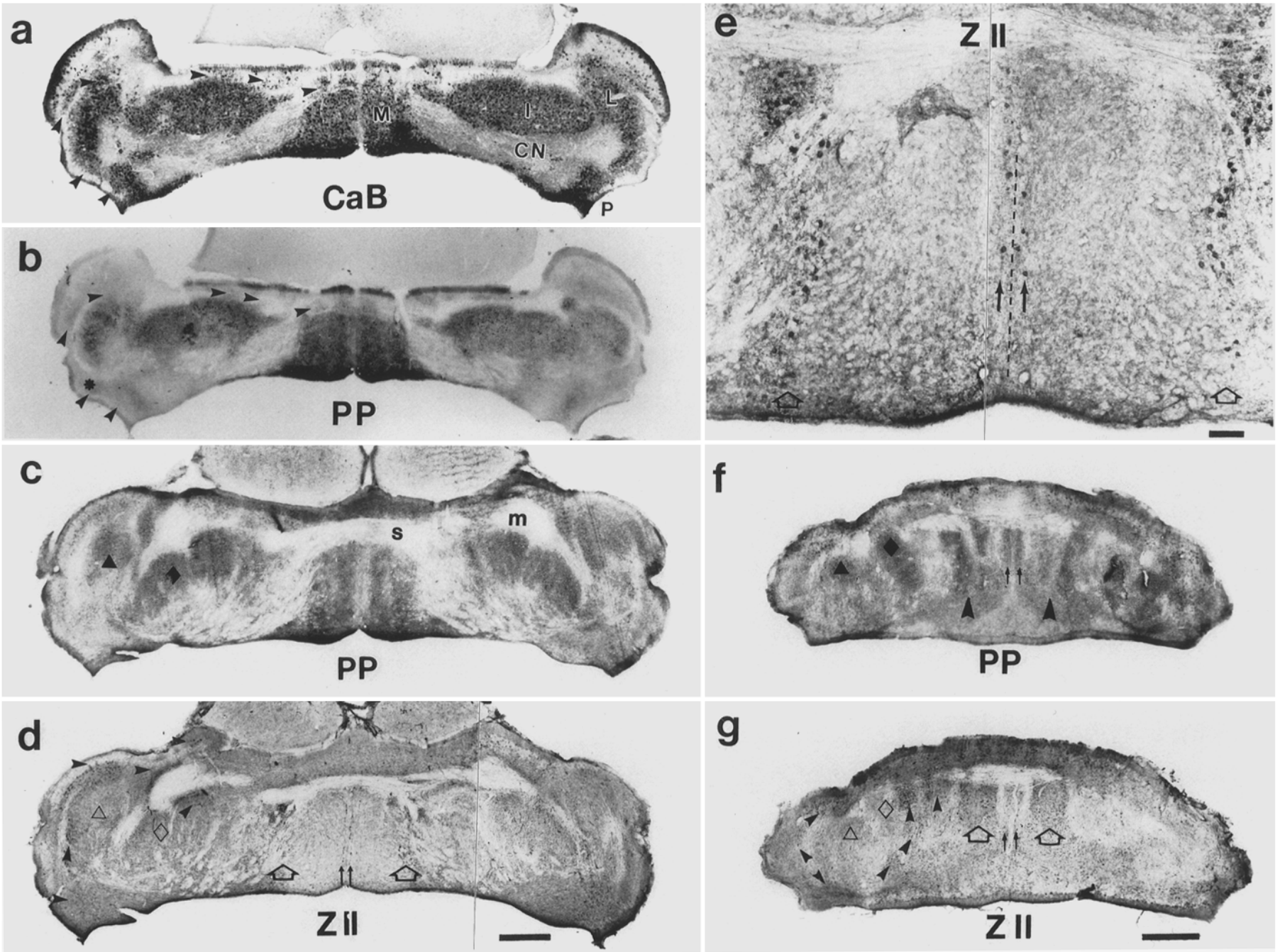

Fig. 2 (for legend see page 422)

immunoreactivity was present within individual cells also intensely stained with the nickel-black DAB reaction product associated with P-path immunoreactivity, the presence of brown reactivity within the coarse dendritic processes surrounding cells with black reaction product was used to classify a Purkinje cell zone as staining with both antibodies (PP&Z, Fig. 3a). In some double-stained zones, individual somata display exclusively the brown DAB reaction product indicative of ZII immunoreactivity (Fig. 3b, arrows).

The computer graphic reconstruction was based on charts of one case processed for double-immunocytochemical staining, with Zebrin II staining zones rendered in blue, P-path positive zones colored yellow and zones with dual staining demarcated in green. The 3D reconstruction is illustrated by surface views of the subcortical masses shown from caudal, dorsal and rostral perspectives (Fig. 4b, c, d, respectively) and by an interior view of a thick slice shown from a caudal perspective (Fig. 4a). Important aspects of the antigenic compartmentation patterns are clarified by these views. The antigenic zones, viewed from a dorsal perspective (Fig. 4c), tend to show an elongated configuration in the rostrocaudal dimension. Like the bands in the normal cerebellar cortex, the P-path reactive zones increase in width in rostral cerebellum, and, conversely, Zebrin II stained zones become broader in caudal cerebellum. This appearance of rostrocaudally elongated zones is partially obscured in surface views of the subcortical masses because of superficial sheet-like zones of Zebrin II immunoreactivity. For example, a swath of Zebrin II stained Purkinje cells extends mediolaterally along the dorsal surface at central cerebellar levels and contributes to the bridges of continuity between the medial, intermediate and lateral divisions of the subcortical masses (see Fig. 4a, c in the region rostral to the sites labeled a, b). Also, a thin sheet of Zebrin II staining extends over the caudal surface of the medial cell mass, and, consequently, the zonal pattern more rostrally is obscured in views rendered from a caudal perspective (Fig. 4b, asterisks).

Approximately seven elongated antigenic zones may be discriminated by inspection of the reconstruction (e.g. Fig. 4c). In this estimate, the thin bands labeled 1 and 5, judged to show dual labeling by our charting criteria, are considered as distinct zones, and the band labeled 3 is considered as a single largely Zebrin II-immunoreactive-zone that shows a partial overlap with P-path-reactive

Fig. 2a–g. Purkinje cell immunostaining patterns in 4-week-old and adult reeler mutants. **a, b** Mid-cerebellar transverse sections from a juvenile mutant stained with calbindin (**a**) and P-path (**b**) antibodies. Subcortical Purkinje cell masses exhibit medial, intermediate and lateral subdivisions, labeled *M*, *I*, and *L*, respectively. The lateral mass includes a ventrolateral region considered homologous to the floccular/paraflocculer lobe in wild-type mice (*P*). *Matching arrowheads* in the two panels point to sites where Purkinje cells stain with calbindin but do not show above background labeling with P-path antibodies. The sites of the deep cerebellar nuclei (*CN*) do not show cellular labeling, but only a light reactivity consistent with reactivity in corticonuclear axons. **c,d** Comparable sections from an adult reeler mutant stained with P-path and Zebrin II antibodies, respectively. Note the extensive expression of P-path in the medial cell mass, which at this level is separated from the overlying cortex by the white matter of the superior cerebellar peduncle (labeled *s*), and in the intermediate cell mass (marked with a *diamond* in **c** and **d**), which lies below fibers of the middle cerebellar peduncle (*m*). The patch of P-path staining in the lateral Purkinje mass (marked with a *triangle*) is flanked by Zebrin II-reactive cell clusters. These and other Zebrin II-positive Purkinje cell groups are denoted with *arrowheads* in **d**. The thin band of Zebrin II staining on each side of the midline (*small arrow*) is barely resolvable at this magnification due to the background staining in this section. **e** Enlarged view of medial Purkinje cell mass from a Zebrin II stained section. Reactive cells are located in a thin vertical band along the midline (*small arrows*) and at the lateral boundary of the medial mass (*open arrows*). **f, g** Sections through the caudal cerebellum of the adult mutant stained with P-path and Zebrin II antibodies, respectively. *Small arrows* point to a peri-midline band in the medial cell mass which stains with both antibodies. The *open arrows* in **g** indicate broad bands of Zebrin II staining more laterally in the medial cell mass. P-path-positive and Zebrin II-negative zones of the intermediate and lateral cell masses are marked with *diamonds* and *triangles*, respectively. *Arrowheads* in **g** point to concentrations of Zebrin II-reactive cells in the intermediate and lateral masses. *Bars* 500 μm in **a–d** and **f,g** and 100 μm in **e**

Fig. 3a, b. Examples of single-labeled and dual-labeled zones from reeler cerebellar sections processed for combined immunocytochemical labeling with P-path and Zebrin II antibodies. **a** Color microphotographic view of the dorsal transition region between the medial and intermediate cell masses and overlying cortex at a central cerebellar level. On the left, the zone marked *PP & Z* may be seen to contain Purkinje cells labeled black with P-path immunoreactivity (nickel-DAB reaction product) and a cloud of coarse dendritic processes labeled brown with Zebrin II immunoreactivity (DAB reaction product). The adjacent Purkinje cell group (*Z*) shows only brown reactivity in cells and processes and thus corresponds to a zone reactive only for the Zebrin II antigens. **b** Transverse sector through the dorsal transition region between the intermediate and lateral subcortical Purkinje cell masses. Zones with staining by Zebrin II alone, by Zebrin II and P-path, and by P-path alone are evident (labeled *Z*, *PP & Z*, and *PP*, respectively). In the zone with dual staining, some individual somata show only the brown DAB reaction product corresponding to Zebrin II immunoreactivity (e.g. *small arrows*). *Bar* 100 μm

Fig. 4a–d. Computer graphic reconstruction of antigenic zones in the subcortical Purkinje cell masses of an adult reeler mouse. *Blue zones* are positive only for Zebrin II, *yellow zones* are positive only for P-path, and *green zones* exhibit reactivity for both antibodies. **a** Central slice 280 μm thick rendered from a caudal viewpoint. Based on charting of five out of a series of eight sections. Outlines of the superficial boundaries from the first and last sections are shown with *pink lines*. The three major subdivisions of the Purkinje cell masses are readily visualized (labeled *M*, *I*, and *L*). Antigenic zones with a substantial extension in the dorsoventral axis are labeled 1–9. The sites labeled **a** and **b** correspond to the location of photographic enlargements shown in Fig. 3a and 3b, respectively. **b, c, d** Caudal, dorsal and rostral views, respectively, of the complete reconstruction (minus a part of the most rostrolateral cerebellum, which was lost in processing). Rostral is *down* in panel **c**. Note that Zebrin II-positive zones predominate caudally and P-path-positive zones rostrally. About seven antigenic zones may be distinguished on the basis of major extension in the rostrocaudal axis (labeled 1–7 in **c**). These patterns are partially obscured by superficial sheets of Zebrin II immunoreactivity that extend mediolaterally between points labeled **a** and **b** in panel **c** (same points as in panel **a**). The *asterisks* in **b** mark a sheet-like extension of Zebrin II immunoreactivity that covers the caudal surface of the large P-path-reactive zones of the medial cell mass. *Bar* 500 μm

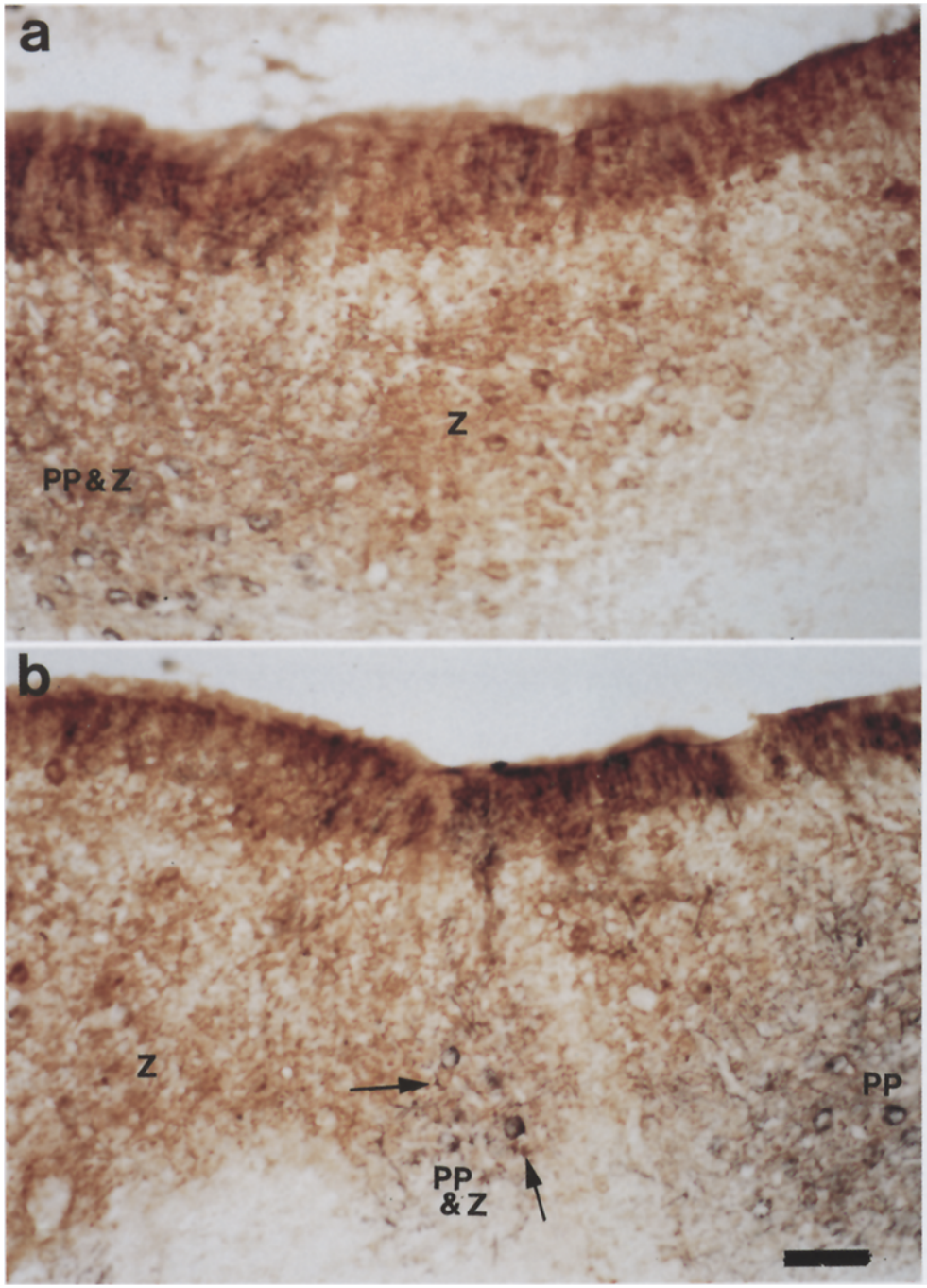

Fig. 3a, b

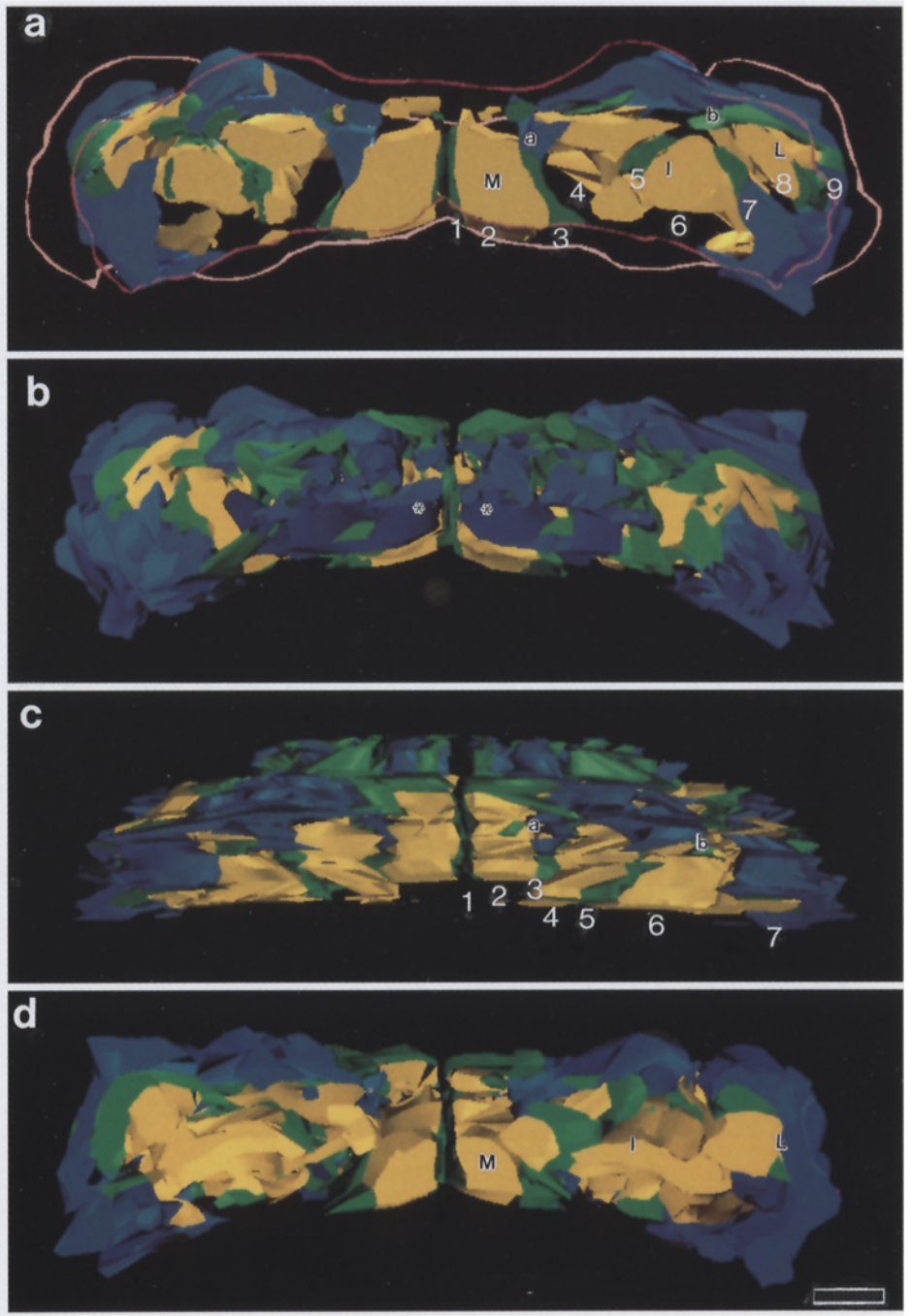

Fig. 4a–d

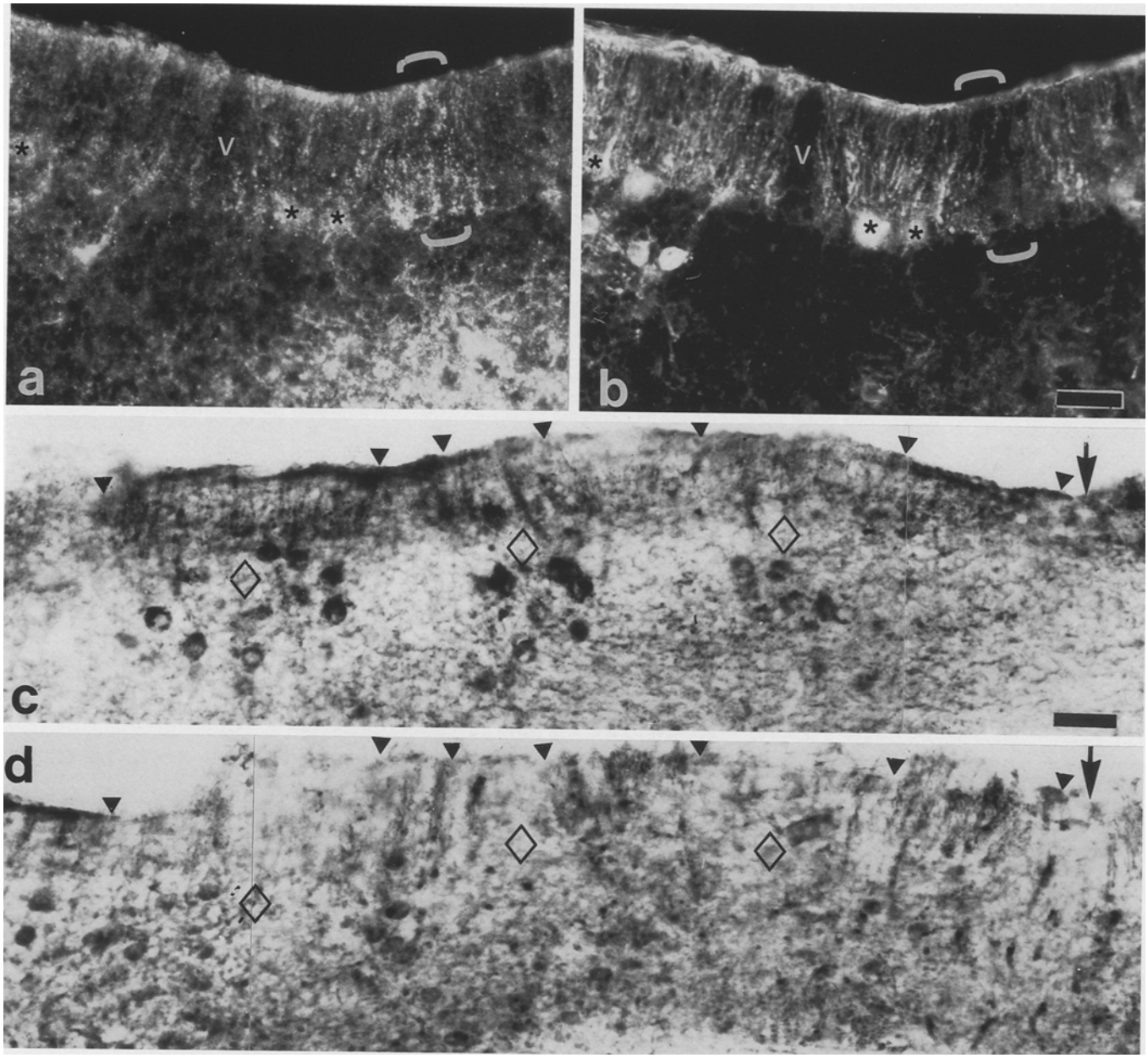

Fig. 5a–d. High magnification views of the cerebellar cortex and subjacent regions of an adult reeler mutant stained with P-path and Zebrin II. **a, b** Double-immunofluorescent staining from an intermediate site in the rostral-caudal and medial-lateral extent of the cerebellum. P-path staining (**a**) labels one patch of cortical Purkinje cells (*brackets*) which lie in a Zebrin II-negative zone (**b**). Other scattered P-path-staining cells are either mixed with ZII^+ cells or show dual labeling (e.g. *asterisks*). A reference blood vessel is labeled with a *v*. **c, d** Alternate sections from caudal cerebellum stained with P-path and Zebrin II antibodies, respectively. Enlarged from Fig. 2f, g. Photographs are aligned at the midline (*arrows* on the right). Three clusters of P-path-positive cells in the cortex and subjacent regions are located at sites that are largely Zebrin-negative (*diamonds*), and vice versa for P-path-negative and Zebrin II-positive zones. *Inverted triangles* mark the approximate boundaries of these antigenically distinct zones. *Bars* 50 μm

zones on each side. When one tries to count antigenic zones with substantial extension in the dorsoventral dimension, perhaps two additional zones in the lateral cerebellum might be considered (Fig. 4a). Aside from the smaller total number of antigenic zones, the relatively few zones detected in the medial cell mass, which is believed homologous to the vermis, contrasts strongly with the banding pattern found in the normal vermis (Fig. 1).

Interpretation of results from the mutant depend on assumptions about the normalcy of representation of antigenic cell types in the subcortical masses. For this reason, the relative volume of zones charted as staining for P-path only, for Zebrin II only, or for both, were quantified using the point-counting stereological method (Table 1). The results bear out the above qualitative observations on rostrocaudal differences in prevalence of antigen expression. For example, in the rostral fourth of the cerebellum, zones with P-path staining only represent 51% of the total volume occupied by Purkinje cells, but in the caudal quarter their volume percent representation

Table 1. Relative volume of zones in subcortical masses immunostainedwith Zebrin II (ZII), P-path (PP) or with both antibodies in the reeler mutant cerebellum

Quartile	%ZII	%ZII + PP	%PP	%Total ZII	%Total PP
Caudal 1	41.4	29.9	28.7	71.3	58.6
2	28.1	15.9	56.0	44.0	71.9
3	22.2	12.0	65.8	34.2	77.8
Rostral 4	35.0	14.1	50.9	49.1	65.0
Means	31.7	18.0	50.3	49.6	68.3

drops to 29%. Conversely, the same measures for Zebrin II stained zones increases from 35% to 41%. The data on zones containing staining for both antigens ranges between 14 and 30%, depending on rostrocaudal level. Although these latter values appear to be high, the determination of the relative representation of dual labeled zones in the normal cerebellar cortex was beyond the scope of the present investigation.

The sparseness of Purkinje cells in the cortical layer of reeler mutants makes it difficult to determine whether they are organized into longitudinal bands. Nevertheless, examination of double- immunofluorescent-labeled sections (Fig. 5a, b) or alternate sections stained with Zebrin II and P-path (Fig. 5c, d) reveals that the molecular layer of the mutant shows significant segregation among dendrites stained with the two antibodies. These dendrites arise from Purkinje cell somata in the cortex and from somata located more deeply in either the granule cell layer or in superficial regions of the subcortical cell masses. The territories occupied by both types of dendritic staining exhibited an alternating pattern with intervening zones of overlap. Such territories extend in the rostrocaudal dimension over at least three to four sections.

Discussion

The parasagittal compartmentation of the cerebellum poses a major challenge for understanding how such an organizational pattern arises in development. The present results with Zebrin II and P-path immunostaining in the reeler mutant provide not only new information on phenotypic abnormalities of the cerebellum, but they also bear on the factors that determine biochemical heterogeneity of normal Purkinje cells.

Despite the premature termination of Purkinje cell migration beneath the white matter, the cells that form the ectopic masses do become organized into alternating zones that stain with Zebrin II, P-path or with both antibodies. Moreover, these antigenic zones are generally oriented in the sagittal plane. Segregated zones of antigen expression spaced by bands of mixed expression were also evident for Purkinje dendrites in the molecular layer. These arise both from the sparse cell population located appropriately in the cortex and from somata in the granule cell and subcortical masses.

Because of the nearly complete absence of foliation in the reeler cerebellum, it is difficult to make explicit comparisons with the parasagittal zonation of the cerebellum in wild type mice. A similar feature in the organization of antigen distribution patterns of both wild type and reeler mice is a tendency for wider P-path staining cell groups in rostral cerebellum and for wider Zebrin II stained zones in caudal cerebellum. One obvious difference is that there are fewer zones of Purkinje cells within the subcortical masses than in the normal adult cortex: roughly 7 to 9 zones are revealed per side in the mutant compared with 14 major divisions in wild-type mice. This difference is particularly evident in the medial subcortical mass, which may reasonably be considered as the homologue of the vermis in wild type mice. We detected on each side a thin dual-stained zone near the midline, a large P-path-labeled zone, and small flanking zones with Zebrin II staining which partially overlapped the P-path-positive zone. By contrast, the normal vermis exhibits bilaterally three Zebrin-positive and three Zebrin-negative bands, of which the third Zebrin-reactive band and the Zebrin II-negative bands stain with the P-path antibody.

Previous observations by others support the inference that the number of longitudinal zones in the subcortical Purkinje population is abnormally small. In one study, Purkinje cell immunoreactivity for the protein L7 was shown to define about five zones unilaterally in the neonatal mouse vermis, but in reeler mutants during the same period the entire medial cell mass was stained (Smeyne et al. 1991). Photographs of staining with 5′-nucleotidase in the reeler mouse (Eisenman 1988) and of anterograde labeling of climbing fiber innervation (Goffinet et al. 1984, Blatt and Eisenman 1988) reveal a small number of zonal subdivisions in the medial cell mass, apparently two or three on each side. In each of these studies, a band through the body of the medial cell mass is evident, which contrasts with the P-path staining pattern observed in the present study. However, Zebrin II staining was abnormally weak in the ectopic Purkinje cells of the mutant, and the presence of heterogeneities in the density of P-path staining within the medial mass raises the possibility of an additional Purkinje cell zone (cf Fig. 2c, f). Nevertheless, it is fair to conclude that there are fewer longitudinal zones in the medial cell mass of the mutant than in the normal vermal cortex.

The present results have implications for understanding how Purkinje cell compartmentation arises in development. Quite different classes of mechanism may be distinguished by experimentally determining the stage at which cellular patterning emerges in development. If the cell heterogeneity that underlies compartmental organization exists prior to postmigratory cell assembly, then one must look at the events of specification in the ventricular zone and regulation of cell migration for the origins of pattern formation. Alternatively, if the cells acquire their divergent properties after migration, then one must also consider either cooperative interactions among the cell populations during differentiation or inductive influences from afferents already organized in the appropriate pattern. Since cell migration of most Purkinje cells is aborted subcortically in the mutant, the presence of segregation among groups of cells expressing different antigens suggests, though does not prove, that neurons des-

tined to express these markers are segregated during their migration.

In previous developmental studies in the rat and mouse, the mature banded pattern of Zebrin immunoreactivity emerges only during the 3rd week postnatally (Leclerc et al. 1988; Tano et al. 1992), which is subsequent to the establishment of normal topographical organization of afferent systems (Sotelo et al. 1984; Arsenio-Nunes and Sotelo 1985; Chedotal and Sotelo 1992; Wassef et al. 1992). However, results of lesion and heterotopic transplantation experiments suggest that Zebrin expression patterns do not depend on the organizing influences of appropriate afferent inputs (Leclerc et al. 1988; Wassef et al. 1990). Also, transplants of dissociated and reaggregated fetal Purkinje cells show patchiness with respect to compartment markers (Rouse and Sotelo 1990), which provides some support for the view that compartmental cell fate is determined prenatally. Better evidence in favor of the latter hypothesis comes from studies demonstrate that biochemical heterogeneities among slab-like groups of Purkinje cells are present at a prenatal period when they are still migrating. For example, discontinuous patterns of staining with antibodies to cyclic GMP protein kinase, calbindin, and L7 have been shown to be transiently present among varying sets of Purkinje cells in late fetal and early postnatal rodents (Wassef and Sotelo, 1984; Wassef et al. 1985; Smeyne et al. 1991). Unfortunately, there is no clear evidence that these early forms of compartmentation are related to the anatomical divisions delineated with Zebrin antibodies in the adult. A correlation has recently been made in the rat between discontinuities in Purkinje cell expression of the protein PEP19 and projections of a subset of climbing fiber afferents reactive with a parvalbumin antibody (Wassef et al. 1992); however, the match was made in one cerebellar lobule only and only during a neonatal period after migration was complete. The consistent finding of studies using other markers for Purkinje cell subsets in the late fetal period is that cluster number is less than that revealed by Zebrin staining at maturity. Careful analysis by Wassef and Sotelo (1984) led them to identify nine zones of positive and negative immunoreactivity for cyclic GMP protein kinase. Interestingly, this is similar to the number of Purkinje slabs revealed by Zebrin II and P-path staining in the adult reeler observed in the present study.

As one possible explanation for reduced compartment number in both the adult reeler and late fetal wild type mouse, we propose that the final phase of migration into the cerebellar cortex might normally involve events that increase the degree of interdigitation between cell sets expressing the two antigens. For example, migratory sets of Purkinje cells destined to express a particular compartment-specific antigen might normally divide into two groups or cause a complementary set of Purkinje cells to split as one group becomes assembled with respect to earlier populations in the emergent cortex. Such a process is suggested from observations by Wassef and Sotelo (1984) of an apparent insertion of a cGMP kinase-positive cell group into a zone of non-staining Purkinje cells in the developing cerebellar cortex (Group III in their Fig. 5).

Alternatively, the Purkinje cells halted in their migration in reeler mutants could correspond to subsets that would normally populate only specific compartments, whereas the cells that form a relatively normal cortex may represent subsets less susceptible to the mutation. If the latter were true, one would expect major differences in the representation of compartment markers in subcortical versus cortical positions, which does not seem to be the case. For example, as presented in Table 1, zones with cells expressing only the Zebrin II antigen occupied 32% of the subcortical masses, a value which is similar to estimates that 30% of all Purkinje cells express the antigen in the normal adult rat (Brochu et al. 1990). Although another 18% of the Purkinje masses corresponds to mixed antigen expression, the lack of counterstaining did not permit assessment of the fraction of Zebrin II-positive cells in these regions. Despite such uncertainty, Zebrin II-stained cells were readily identified in the cortex, often in apparent spatial register with Zebrin II-reactive zones in the subcortical masses. Thus, it seems unlikely that the estimated 5% of the Purkinje cell population that resides in the mutant cortex (Heckroth et al. 1989), even if composed of cells that would normally populate a subset of Purkinje cell zones, could account for missing bands in the subcortical population.

A final possibility is raised by the additional finding of Heckroth et al. that total Purkinje cell number in the mutant is 40–50% less than normal. Thus, it is conceivable that the reeler mutation causes the preferential death of cell groups that would normally form additional zones of each label type. However, compared to the hypothesis we favor, such an explanation does not account for the similarity in number of Purkinje cell zones detected subcortically in the adult reeler mouse and in the normal rodent during embryogenesis. Also, this explanation requires extra assumptions difficult to reconcile with evidence from mutant wild-type chimeras (Mullen 1977, Terashima et al. 1986). The presence of wild-type cells in such genetic mosaics allows cerebellar development to proceed normally, indicating that the mutant phenotype is not autonomously expressed within all or subsets of the Purkinje cell population but instead arises from a deficient interaction of Purkinje cells with their environment. Thus, it is simpler to presume that the two mutant phenotypes of reduced cell number and reduced number of antigenic compartments are independent effects, both secondary to the deficiency in cell migration, than to posit a selective effect of the mutant gene on survival promoting interactions of Purkinje cell subsets with their environment.

The present observations on Purkinje cell compartmentation in the reeler mutant mouse contribute to a growing body of evidence that cell fate with respect to these compartments is determined at a very fundamental level. For example, the expression of a number of transgenes inserted into the mouse genome has been shown to conform to a longitudinal banding pattern (see Oberdick et al, 1993, for discussion). Also, Purkinje cell death caused by three mutations in the mouse, nervous (*nr*), Purkinje cell degeneration (*pcd*), and tambaleante (*tbl*), has been shown to proceed with a preferential rate or extent among one set of Purkinje cell bands (Wassef et al.

1987). In the nervous mutant, cell bands surviving in the vermis correspond closely with the Zebrin negative and P-path positive Purkinje cell compartments (Wassef et al. 1987; Edwards et al. 1994). As is the case with the *reeler* gene, it is unclear whether or not the effects of transgene insertion or of these single gene mutations on the parasagittal banding pattern reflect a direct interaction with the genes that are fundamentally involved in regulating compartmental heterogeneity. However, our finding of a significant degree of antigenic compartmentation among Purkinje cells that fail to complete their migration provides support for the hypothesis that the critical genetic expression patterns that lead to antigenic compartmentation in normal development begin to operate before or during cell migration.

Acknowledgements. This work was supported by NIH Grants HD21018 (M.A.E.), NS24386 (J.E.C.), NS18381 (N.L.), and HD05515 (M.Y.), and by the Massachusetts Department of Mental Health (contract no.100220023SC). We thank Patricia Harlan for technical assistance, Denise Brescia for typing, Charles Gerfen for calbindin antibody, and Richard Hawkes for Zebrin II antibody and for helpful discussions.

References

Arsenio-Numes, Sotelo C (1985) Development of the spinocerebellar system in the postnatal rat. J Comp Neurol 237:291–306

Blatt GJ, Eisenman LM (1988) Topographic and zonal organization of the olivocerebellar projection in the reeler mutant mouse. J Comp Neurol 267:603–615

Boegman RJ, Parent A, Hawkes R (1988) Zonation in the rat cerebellar cortex: patches of high acetyl-cholinesterase activity in the granular layer are congruent with Purkinje cell compartments. Brain Res 448:237–251

Brochu G, Maler L, Hawkes R (1990) Zebrin II: a polypeptide antigen expressed selectively by Purkinje cells reveals compartments in rat and fish cerebellum. J Comp Neurol 291:538–552

Buisseret-Delmas C, Angaut P (1993) The cerebellar olivo-corticonuclear connections in the rat. Prog Neurobiol 40:63–87

Caviness VS Jr, Crandall JE, Edwards MA (1988) The reeler malformation: implications for neocortical histogenesis. In: Peters A, Jones EG (eds) Cerebral cortex vol7. Plenum Press, New York, pp 159–89

Chedotal A, Sotelo C (1992) Early development of olivocerebellar projections in the fetal rat using CGRP immunohistochemistry. Eur J Neurosci 4:1159–1179

Chou DKH, Flores S, Jungalwala FB (1990) Identification of disialosyl paragloboside and O-acetyldisialosyl paragloboside in cerebellum and embryonic cerebrum. J Neurochem 54:1598–1607

Edwards MA, Crandall JE, Leclerc N, Yamamoto M (1994) Effects of *nervous* mutation on Purkinje cell compartments defined by Zebrin II and 90-acetylated gangliosides expression. Neurosci Res 19:167–174

Eisenman LM (1988) Histochemical localization of 5′-nucleotidase in the reeler mutant mouse. Neurosci Lett 94:70-75

Eisenman LM, Hawkes R (1989) 5′-Nucleotidase and the mabQ113 antigen share a common distribution in the cerebellar cortex of the mouse. Neuroscience 31:231–235

Eisenman LM, Hawkes R (1993) Antigenic compartmentation in the mouse cerebellar cortex: Zebrin and HNK-1 reveal a complex, overlapping molecular topography. J Comp Neurol 335:586–605

Goffinet AM (1983) The embryonic development of the cerebellum in normal and reeler mutant mice. Anat Embryol 168:73-86

Goffinet AM (1984) Events governing organization of postmigratory neurons: studies on brain development in normal and reeler mice. Brain Res Rev 7:261–296

Goffinet AM, So K-F, Yamamoto M, Edwards M, Caviness VS Jr (1984) Architectonic and hodological organization of the cerebellum in reeler mutant mice. Dev Brain Res 16:263–76

Gravel C, Hawkes R (1990) Parasagittal organization of the rat cerebellar cortex: direct comparison of Purkinje cell compartments and the organization of the spinocerebellar projection. J Comp Neurol 291:79–102

Gravel C, Eisenman LM, Sasseville R, Hawkes R (1987) Parasagittal organization of the rat cerebellar cortex: direct correlation between antigenic Purkinje cell bands revealed by mabQ113 and the organization of the olivocerebellar projection. J Comp Neurol 265:294–310

Hancock MB (1986) Two-color immunoperoxidase staining: visualization of anatomic relationships between immunoreactive neural elements. Am J Anat 175:343–352

Hawkes R (1992) Antigenic markers of cerebellar modules in the adult mouse. Biochem Soc Trans 20:391–395

Hawkes R, Leclerc N (1986) Immunocytochemical demonstration of topographic ordering of Purkinje cell axon terminals in the fastigial nuclei of the rat. J Comp Neurol 244:481–491

Hawkes R, Leclerc N (1987) Antigenic map of the rat cerebellar cortex: The distribution of parasagittal bands as revealed by monoclonal anti-Purkinje cell antibody mabQ113. J Comp Neurol 256:29–41

Hawkes R, Colonnier M, Leclerc N (1985) Monoclonal antibodies reveal sagittal banding in the rodent cerebellar cortex. Brain Res 333:359–365

Heckroth JA, Goldowitz D, Eisenman LM (1989) Purkinje cell reduction in the reeler mutant mouse: a quantitative immunohistochemical study. J Comp Neurol 279:546–555

Inoue Y, Maeda N, Kokubun T, Takayama C, Inoue K, Terashima T, Mikoshiba K (1990) Architecture of Purkinje cells of the reeler mutant mouse observed by immunohistochemistry for the inositol 1,4,5-trisphosphate receptor protein P400. Neurosci Res 8:189–201

Leclerc N, Gravel C, Hawkes R (1988) Development of parasagittal zonation in the rat cerebellar cortex: MabQ113 antigenic bands are created postnatally by the suppression of antigen expression in a subset of Purkinje cells. J Comp Neurol 273:399–420

Leclerc N, Dore L, Parent A, Hawkes R (1990) The compartmentation of the monkey and rat cerebellar cortex: Zebrin I and cytochrome oxidase. Brain Res 506:70–78

LeClerc N, Schwarting G, Herrup K, Hawkes RB, Yamamoto M (1992) Compartmentation in mammalian cerebellum: Zebrin II and P-path antibodies define three classes of sagittally organized bands of Purkinje cells. Proc Nat Acad Sci USA 89:5006–5010

Mariani J, Crepel F, Mikoshiba K, Changeux JP, Sotelo C (1977) Anatomical, physiological and biochemical studies of the cerebellum from reeler mutant mouse. Philos Trans R Soc Lond Biol 281:1–28

Matsushita M, Ragnarson B, Grant G (1991) Topographic relationship between sagittal Purkinje cell bands revealed by a monoclonal antibody to Zebrin I and spinocerebellar projections arising from the central cervical nucleus in the rat. Exp Brain Res 84:133–141

Meier H, Hoag WG (1962) The neuropathology of "reeler", a neuromuscular mutation in mice. J Neuropath Exp Neurol 21:649–654

Mullen RJ (1977) Genetic dissection of the CNS with mutant-normal mouse and rat chimeras. Soc Neurosci Symp 2:47–65

Oberdick J, Shilling K, Smeyne RJ, Corbin JG, Bocchiaro C, Morgan JI (1993) Control of segment-like patterns of gene expression in the mouse cerebellum. Neuron 10:1007–1018

Pinto-Lord MC, Evrard P, Caviness VS Jr (1982) Obstructed neuronal migration along radial glial fibers in the neocortex of the reeler mouse: a Golgi-EM analysis. Dev Brain Res 4:379–393

Rouse RV, Sotelo C (1990) Grafts of dissociated cerebellar cells containing Purkinje cell precursors organize into Zebrin I defined compartments. Exp Brain Res 82:401–407

Scott TG (1964) A unique pattern of localization within the cerebellum of the mouse. J Comp Neurol 122:1–8

Smeyne RJ, Oberdick J, Schilling K, Berrebi AS, Mugnaini E, Morgan JI (1991) Dynamic organization of developing Purkinje cells revealed by transgene expression. Science 254:719–721

Sotelo C, Wassef M (1991) Purkinje cell heterogeneity in four cerebellar mutations revealed by Zebrin I expression. Neurosci Abst 17:918

Sotelo C, Bourrat F, Triller A (1984) Postnatal development of the inferior olivary complex in the rat. II. Topographic organization of the immature olivocerebellar projection. J Comp Neurol 222:177–199

Tano D, Napieralski JA, Eisenman LM, Messer A, Plummer J, Hawkes R (1992) Novel developmental boundary in the cerebellum revealed by Zebrin expression in the Lurcher (*Lc*/+) mutant mouse. J Comp Neurol 323:128–136

Terashima T, Onoue K, Inoue Y, Yokoyama M, Mikoshiba K (1986) Observations on the cerebellum of normal-reeler mutant mouse chimera. J Comp Neurol 252:264–278

Underwood EE (1970) Quantitative stereology. Addison-Wesley Press, Reading, Pa

Voogd J (1969) The importance of fiber connections in the comparative anatomy of the mammalian cerebellum. In: Llinas R (ed) Neurobiology of cerebellar evolution and development. AMA, Chicago, pp 493–541

Voogd J, Gerrits NM, Marani E (1985) Cerebellum. In: The rat nervous system. vol 2. Academic Press, Sydney, pp 251–291

Wassef M, Sotelo C (1984) Asynchrony in the expression of guanosine 3′:5′-phosphate-dependent protein kinase by clusters of Purkinje cells during the perinatal development of rat cerebellum. Neuroscience 13:1217–1241

Wassef M, Zanetta JP, Brehier A, Sotelo C (1985) Transient biochemical compartmentalization of Purkinje cells during early cerebellar development. Dev Biol 111:129–137

Wassef M, Sotelo C, Cholley B, Brehier A, Thomasset M (1987) Cerebellar mutations affecting the postnatal survival of Purkinje cells in the mouse disclose a longitudinal pattern of differentially sensitive cells. Dev Biol 124:379–389

Wassef M, Sotelo C, Thomasset M, Granholm A-C, Leclerc N, Rafrafi J, Hawkes R (1990) Expression of compartmentation antigen Zebrin I in cerebellar transplants. J Comp Neurol 294:223–234

Wassef M, Cholley B, Heizmann CW, Sotelo C (1992) Development of the olivocerebellar projection in the rat. II. Matching of the developmental compartmentations of the cerebellum and inferior olive through the projection map. J Comp Neurol 323:537–550

Wilson L, Sotelo C, Caviness VS Jr (1981) Heterologous synapses upon Purkinje cells in the cerebellum of the reeler mutant mouse: an experimental light and electron microscopic study. Brain Res 213:63–82

Yuasa S, Kitoh J, Oda S, Kawamura K (1993) Obstructed migration of Purkinje cells in the developing cerebellum of the reeler mutant mouse. Anat Embryol 188:317–329