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Compartmentation in mammalian cerebellum: Zebrin II and P-path antibodies define three classes of sagittally organized bands of Purkinje cells

(cerebellar cortex/parasagittal bands/acetylated gangliosides)

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ABSTRACT **The respective roles of genetic and epigenetic factors in generation of pattern formation in the vertebrate nervous system are still poorly elucidated. The mammalian cerebellum is subdivided in parasagittal modules defined by anatomical, physiological, and biochemical criteria. Immunostaining of adult mouse cerebellum with two monoclonal antibodies, P-path, which recognizes 9-O-acetylated glycolipids, and Zebrin II, which recognizes a 36-kDa protein, reveals three classes of sagittally organized bands of Purkinje cells: two complementary groups distinctly immunoreactive to one antibody but not the other and a third group that contains double-labeled cells. No Purkinje cells could be detected that were unreactive to either antibody. The specific and reproducible topography of these three classes of Purkinje cells may be related to the compartmentation of the cerebellum into developmental genetic modules.**

Cell patterning in any organism is generated by a series of pattern formation events that are controlled in both time and space by a network of interacting genes. One of the more intriguing types of pattern, encountered in a variety of species, is one in which there is an apparent periodicity to the pattern element. The cerebellum is a valuable model system in which to study pattern development in the vertebrate central nervous system. Despite the uniform morphological appearance of the cerebellar cortex, a variety of molecular, biochemical, physiological, and anatomical criteria can be used to subdivide the cerebellum into a series of longitudinally organized bands. Eloquent examples of this organization are the Zebrin I (ZI) and Zebrin (ZII) antigens that are confined to a subset of Purkinje cells (1, 2). ZII antigen is a 36-kDa protein expressed by 30% of the rodent cerebellar Purkinje cells (2). The spatial organization of both ZI- and ZII-positive Purkinje cells defines sagittal bands that run in the anterior–posterior dimension (2, 3). Not only is this pattern reproducible from individual to individual, but the number of bands is preserved during phylogeny (4, 5). In mammalian cerebellum, ZII antibody stains on each side of the midline, three bands in the vermis, one at the margin of the vermis and the hemisphere, and three in the hemisphere, separated by interbands of ZII-negative Purkinje cells. There is a close relationship between Zebrin compartments and the segregation of cerebellar afferent terminal fields (6, 7). An identical periodic pattern is also revealed by using several other markers that subdivide the cerebellar cortex into two sets of modules—one corresponding to Zebrin-positive compartments and one corresponding to Zebrin-negative compartments (5, 8, 9). Immunostaining of adult mouse cerebellum with a monoclonal antibody, P-path, that recognizes 9-O-acetylated glycolipids, reveals a clear pattern of sagittally organized bands of Purkinje cells that correspond to ZII-negative bands. In addition, a third class of Purkinje cell bands immunoreactive to both antibodies is found. This segregates the Purkinje cells into three classes of cells: ZII positive/P-path negative, ZII positive/P-path positive and ZII negative/P-path positive, each topographically distributed in the cerebellar cortex.

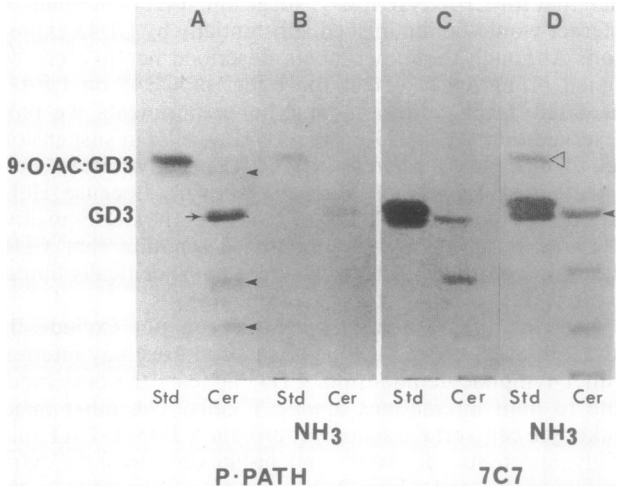

FIG. 1. The acidic glycolipid fraction (gangliosides) from adult mouse cerebellum (Cer) was chromatographed on TLC plates and exposed to P-path and 7C7 antibodies. A mixture of purified GD3 and 9-*O*-acetyl GD3 was used as standard (Std). (*A*) P-path antibody reacts with 9-*O*-acetyl GD3 but not with GD3 in lane Std. P-path reacts with one major cerebellar ganglioside (arrow) and several minor gangliosides (arrowheads). (*B*) Exposure of the chromatogram to ammonia vapors (NH_3) for 15 min after chromatographic development of the gangliosides resulted in loss of P-path-reactive antigens. (*C*) 7C7 antibody reacts with GD3 but not 9-*O*-acetyl GD3 in lane Std. In lane Cer, 7C7 reacts with GD3 and other complex disialogangliosides. (*D*) Ammonia vapor (NH_3) treatment converts standard 9-*O*-acetyl GD3 into a 7C7-reactive ganglioside (open arrowhead), which is probably GD3. The intensity of 7C7 reactivity with cerebellar ganglioside adjacent to GD3 standard (solid arrowhead) also increases because of deacetylation of the major P-path-reactive cerebellar ganglioside.

MATERIALS AND METHODS

Reagents. 7C7 and P-path are both murine monoclonal antibodies (IgM) that react with nonacetylated gangliosides

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Abbreviations: ZI, Zebrin I; ZII, Zebrin II; GD3, disialolactosylceramide.

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(10) and 9-*O*-acetyl gangliosides, respectively (11). ZII, a monoclonal antibody (IgG) that recognizes a protein of 36 kDa, has been produced from a mouse immunized with a crude subcellular membrane fraction from immature rat cerebellum (2).

Immunocytochemistry. Adult C57BL/6 mice were perfused transcardially with 4% paraformaldehyde/0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) followed by overnight immersion of the brain in fixative without glutaraldehyde. Coronal sections of cerebellum were cut 40 μm thick on a freezing stage microtome. On double-stained sections, P-path was revealed by fluorescein isothiocyanate-conjugated secondary anti-mouse immunoglobulin and ZII immunostaining by the Vectastain ABC procedure (Vector Laboratories) using Texas Red as substrate. A three-dimensional reconstruction was performed by a PC-3D (Jandel) system. Serial sections (50 μm) were cut on a cryostat and adjacent sections were stained by antibodies with secondary peroxidase-conjugated anti-mouse immunoglobulin and 4-chloro-1-naphthol as substrate. The drawing of Fig. 4 is based on the PC-3D (Jandal, Corte Medera, CA) reconstruction system.

Glycolipid Analysis. Disialolactosylceramide (GD3) and 9-*O*-acetyl GD3 were purified from bovine buttermilk as described (12) and used as standards for TLC immunostaining experiments. GD3 from buttermilk chromatographs as a double band because of heterogeneity in the lipid portion of the molecule. Glycolipids were extracted from adult mouse cerebellum as described (13). The acidic glycolipid fraction obtained from DEAE chromatography was analyzed by TLC and by TLC immunostaining as described (14). Some chromatograms were exposed to ammonia vapors for 15 min after chromatographic separation of the glycolipids and before exposure to P-path and 7C7 antibodies. This procedure

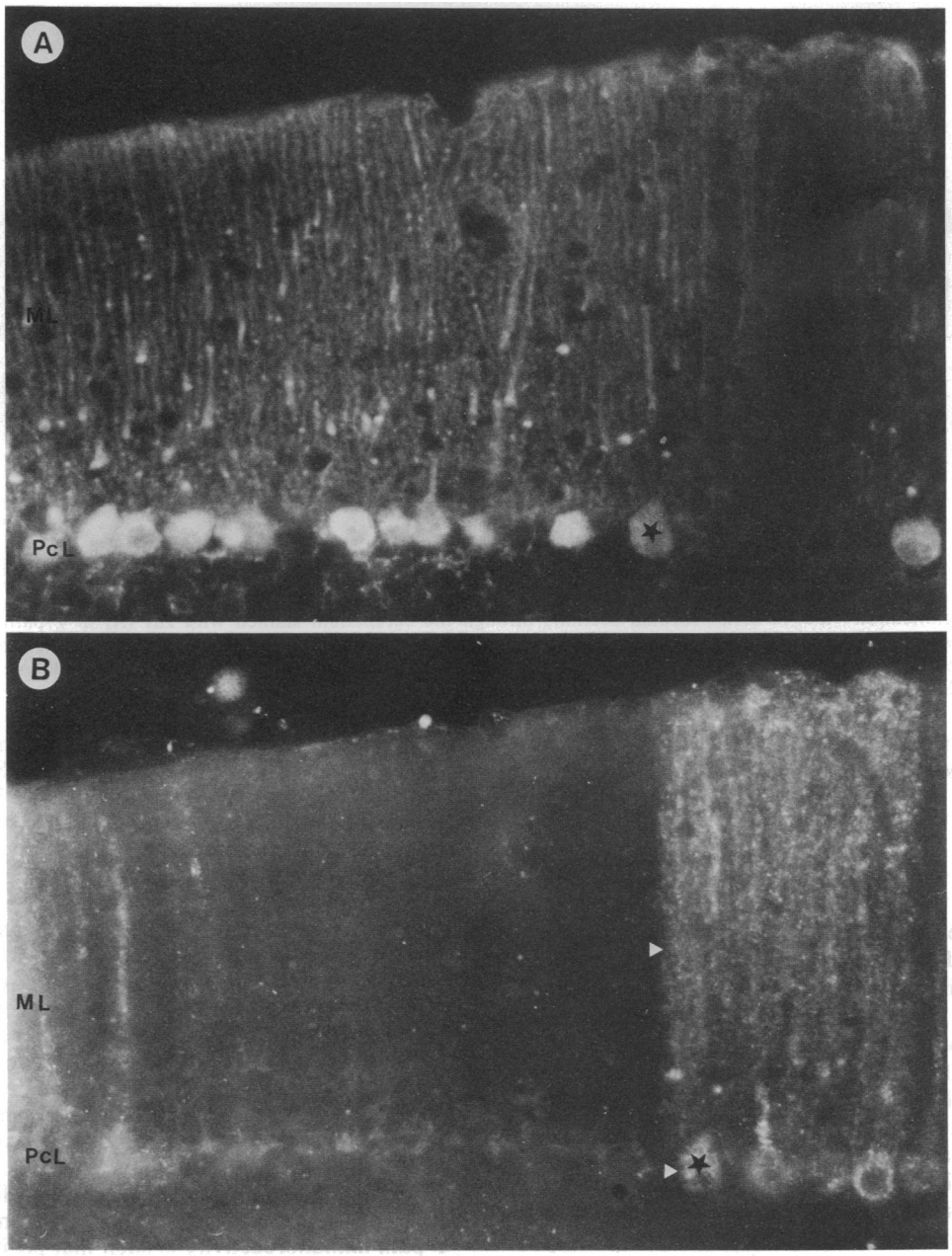

FIG. 2. Photomicrographs of a double-stained coronal section with ZII and P-path antibodies. P-path immunoreactivity was revealed by fluorescein isothiocyanate-conjugated secondary antibody and ZII immunoreactivity by ABC kit conjugated with Texas Red. (*A*) ZII immunoreactivity is located in dendrites (ML) and cell bodies of the Purkinje cells (PcL). (*B*) P-path immunoreactivity was found in both somata (upper arrowhead) and dendrites (lower arrowhead) of Purkinje cells. A double-stained cell is noted at the boundary (★).

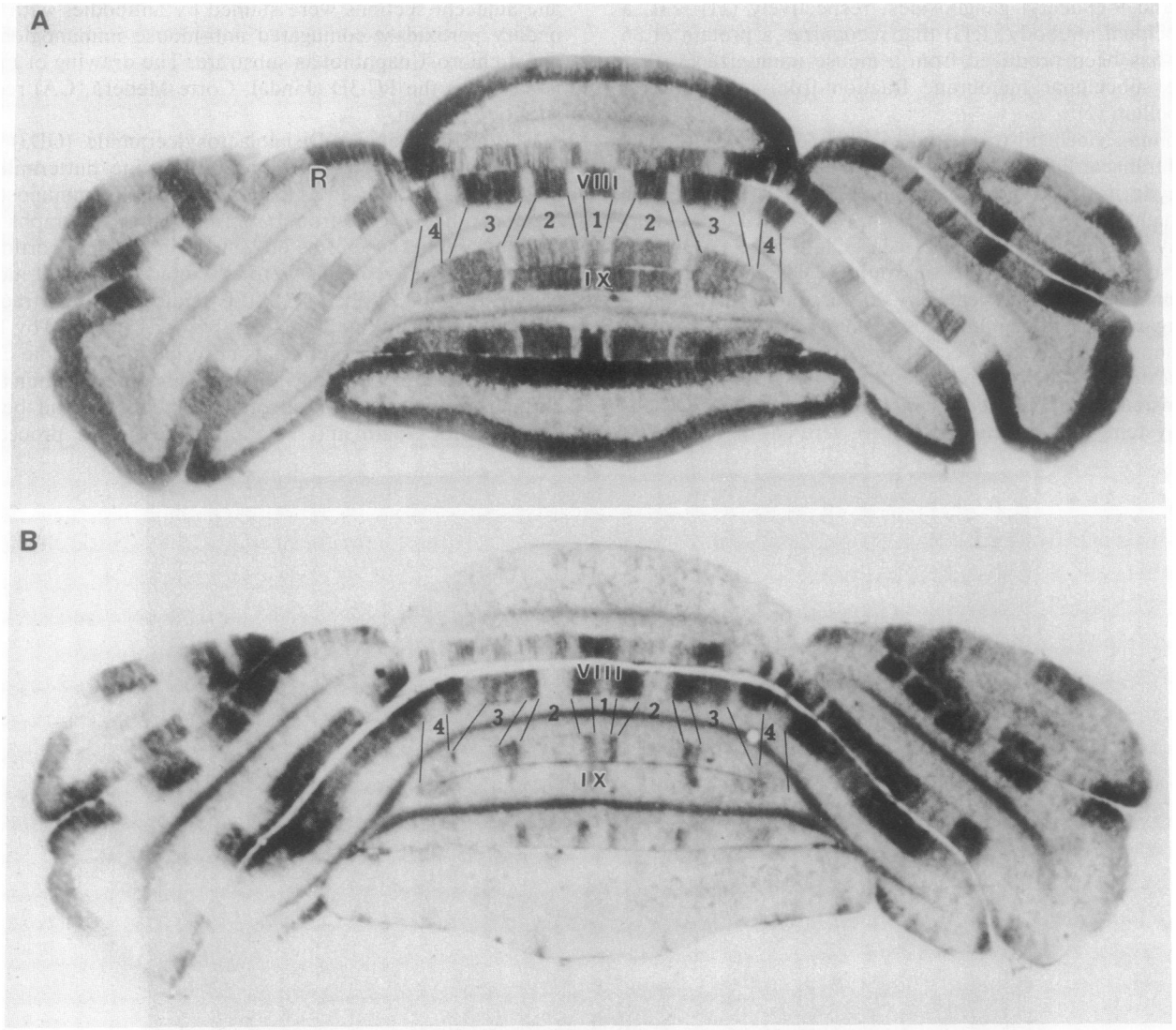

FIG. 3. Low-magnification view of serial coronal sections through the cerebellum stained with ZII (*A*) or P-path antibodies (*B*). The constituent bands are noted according to ref. 3. As revealed by afferent topography, P1 (*1*) is formed by two bands abutted at the midline (7); P2 (*2*) and P3 (*3*) are found more laterally in the vermis; P4 (*4*) is localized at the margin of the vermis and hemisphere; and P3 (*3*) is double labeled in lobule VIII and is only ZII positive in the caudal portion of lobule IX.

converts acetylated gangliosides that react with P-path antibodies to nonacetylated gangliosides reacting with 7C7 antibodies.

RESULTS

On the basis of its chromatographic mobility in adult murine cerebellum, P-path reacts with one major ganglioside likely to be an acetylated derivative of disialolactoneotetraosylceramide and three minor gangliosides (15). Fig. 1 shows characterization of the P-path antibody in comparison with 7C7 antibody. P-path antibody reacts only with 9-*O*-acetyl GD3 (Fig. 1*A*, lane Std). The major P-path immunoreactive ganglioside in the adult cerebellum is not 9-*O*-acetyl GD3 but is likely to be an acetylated derivative of disialolactoneotetraosylceramide (9-*O*-acetyl LM1) (Fig. 1*A*, lane Cer). This acetylated derivative of LM1 that cochromatographs with GD3 was previously characterized from adult mouse cerebellum by Chou *et al.* (15). Chemical deacetylation emphasizes the importance of the acetyl group as part of the P-path epitope. Exposure to ammonia vapors greatly diminishes P-path immunoreactivity with standard 9-*O*-acetyl GD3 and with the cerebellar gangliosides (Fig. 1*B*).

7C7 antibody reacts with GD3 but not with 9-*O*-acetyl GD3 in the standard mixture (Fig. 1*C*, lane Std). In cerebellar extracts, 7C7 antibody reacts with GD3 and three other gangliosides that likely contain disialo terminals. Deacetylation with ammonia reveals a 7C7-reactive ganglioside (Fig. 1*D*, open arrowhead) at the position where 9-*O*-acetyl GD3 was observed. Furthermore, the increase in intensity of the immunoreactive band at the position near standard GD3 (Fig. 1*D*) is due to conversion of the major P-path-reactive ganglioside (as shown in Fig. 1*A*) into a 7C7-reactive ganglioside after removal of the acetyl moiety. We were unable to detect any P-path immunoreactive proteins in adult cerebellum by SDS/PAGE and immunoblotting techniques (data not shown).

Immunocytochemistry of the adult mouse cerebellum shows that P-path staining is primarily associated with Purkinje cell somata and dendrites (Fig. 2*B*). Staining in axons and synaptic terminals in the deep cerebellar nuclei is not as clear as the somatic and dendritic staining. The pattern of P-path immunoreactivity is such that positive Purkinje cells form distinct parasagittal bands separated by negative cells (Figs. 2*B* and 3*B*). P-path immunoreactive bands are no longer observed after deacetylation by exposure of the sections to ammonia vapors.

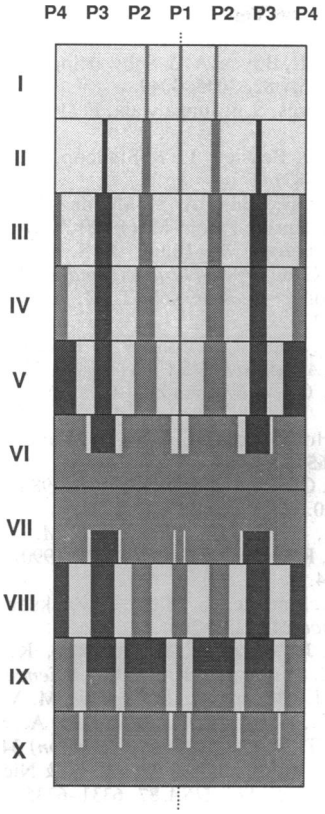

FIG. 4. Dorsal view of a reconstruction of the ZII/P-path staining pattern of the cerebellar vermis. Areas that are ZII positive are lightly shaded; areas that are P-path positive have medium shading; regions that are double labeled are darkly shaded. I–X, cerebellar lobules.

These P-path bands are reminiscent of the compartments revealed with the ZII antibody (Fig. 3). In the vermis, the ZII-immunopositive Purkinje cells are distributed symmetrically in six parasagittal compartments, three per side (P1, P2, and P3), with two (P4) additional bands defining the boundaries between the hemisphere and the vermis (Fig. 3*A*). The bands of ZII-positive Purkinje cells are intercalated by six ZII-negative bands that are immunoreactive to P-path antibody (Fig. 3*B*). The boundaries of two types of Purkinje cells are sharply demarcated, with only one Purkinje cell stained with both antibodies forming these boundaries (Fig. 2). This result supports the conclusion that all Purkinje cells that are ZII negative are P-path positive. P-path thus represents an example of a positive immunological marker for Purkinje cells of the ZII-negative bands. However, the two antigen distributions are not simply complementary. Most notable is the presence of sharply defined double-stained compartments. A complete reconstruction of the staining patterns of the vermis is shown schematically in Fig. 4. The $P3^+$ compartment is both ZII positive and P-path positive throughout the anterior vermis to the rostral portion of lobule IX. In addition, the $P2^+$ compartment in the dorsal region of lobule IX (but nowhere else) is also double labeled. Some regions of the hemispheres are also double labeled (e.g., the $P4^+$ band of lobules V and VIII). We were unable to identify Purkinje cells in any region of the mouse cerebellum that did not stain positively for either the ZII or the P-path antigen.

DISCUSSION

Immunostaining of a cerebellar section with both P-path and ZII permits identification of three classes of Purkinje cells, each with a specific and reproducible topography. This pattern of sagittal zones is reminiscent of the fundamental sagittal zonation of the vertebrate cerebellum. The olivocerebellar projections respect the Zebrin boundaries at most positions (7), as do mossy fiber terminal fields in the granular layer (6). Thus, the regions stained by these antibodies represent compartments of Purkinje cells, each of which comprises a fundamental structural unit of cerebellum.

Acetylated gangliosides have been described in cerebellum (16, 17), but no report has related the staining pattern to the topographical organization of the cerebellar cortex itself. Several glycoconjugates have been described that define different aspects of pattern formation in the developing mammalian nervous system. One of these, 9-*O*-acetyl GD3, is expressed in a dorsal–ventral gradient in the retina of rat at a period in development when retinal ganglion cells are forming topographically organized projections (18). In the dorsal root ganglion, nonoverlapping subsets of neurons express different globoseries and lactoseries glycoconjugates and send processes that terminate in separate laminae of the dorsal horn of the spinal cord (19). It was also shown that specific carbohydrate-binding proteins were coexpressed in lactoseries cells, suggesting that carbohydrate–protein interactions are involved in the guidance of dorsal root ganglion neuronal processes (20). In the olfactory system, glycoconjugates reactive with CC1 and CC2 monoclonal antibodies define subsets of vomeronasal and main olfactory neurons and reveal their axon projection patterns in a manner that suggests their involvement in axon guidance (21). The importance of 9-O-acetylation in the developmental process has recently been emphasized by reports of transgenic mice in which ectopic expression of 9-*O*-acetyl sialic acid esterase under the control of the phenylethanolamine *N*-methyltransferase promoter leads to abnormal removal of 9-O-acetylation and results in early developmental arrest of the retina and adrenal gland of the transgenic embryo (22).

It is likely that the sagittal zonation pattern has a strong developmental genetic basis. The developmental mechanisms underlying cell patterning in the cerebellar cortex are still unknown. Certainly, the available experimental evidence from lesioning (23) and grafting (24) studies suggests that the ultimate ZI phenotype is already independent of afferent inputs from the time of Purkinje cell birth. Several genetic probes including the L7 (25, 26) and rhombotin (27) genes have been described that reveal bands of expression that are oriented, by and large, in the sagittal plane during cerebellar development. Rhombotin genes are also expressed in an alternative segmented pattern in the rhombomeres, suggesting that similar genetic regulatory mechanisms govern both the antero–posterior segmentation of the brainstem and cerebellar sagittal band formation. A recent report (28) has even suggested that certain chromosomal locations (two of six tested) are organized in such a way that inserted transgenes with weak, nonspecific promoters are transcribed in banded patterns in the cerebellum. Finally, the pattern of cell elimination in certain murine neurological mutants such as *nr* (nervous) (29) also appears to be organized according to a system of parasagittal compartments. In addition, the parasagittal banding pattern of the cerebellar input seems to develop independently by being organized in parasagittal bands before they reach their cortical target (30). This reinforces the role of genetic cues in pattern formation of the mammalian cerebellum. The organization of the cerebellar cortex in phenotypically and functionally distinct modules is reminiscent of the cytoarchitectonic areas of the cerebral cortex. A recent study has shown that the genetic program of cells plays a crucial role in differentiation of the cytoarchitectonic areas in primate visual cortex (31).

P-path and ZII both represent one of several qualities that characterize these three Purkinje cell types. The respective function of P-path and ZII antigens in cell patterning and the direct or indirect regulatory mechanisms of pattern expres-

sion between these two molecules during ontogeny remain to be elucidated. As shown previously for ZI, the molecular diversity among Purkinje cells is controlled by intrinsic cellular programs (24). The parasagittal organization of cerebellar cortex may be created by a multiple gene system that is expressed in complementary and overlapping subsets of cells. The topological relationship between P-path, an acetylated ganglioside, and ZII, a protein, reflects the complexity of the developmental programs involved in cell patterning.

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