Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 7131–7135, October 1985
Neurobiology

Diversity among Purkinje cells in the monkey cerebellum

(deep cerebellar nuclei/monoclonal antibody/immunohistochemistry)

VERNON M. INGRAM*, MARILEE P. OGREN†, CLARE L. CHATOT*, JAMIE M. GOSSELS*, AND BARBARA BLANCHARD OWENS*

Departments of *Biology and †Psychology and Brain Science, Massachusetts Institute of Technology, Cambridge, MA 02139

Communicated by Walle J. H. Nauta, June 24, 1985

ABSTRACT **A monoclonal antibody (B1) produced against rat embryonic forebrain membranes shows specific and striking immunohistochemical staining of Purkinje cells in the monkey cerebellum in a pattern of broad parasagittal alternating bands of cells either possessing or lacking the B1 antigen. In addition, the neurons of the deep cerebellar nuclei and some neurons of the motor cortex and of the spinal cord also contain the B1 antigen. Neurons with the B1 antigen were also seen in the somatosensory cortex, the vestibular and cochlear nuclei, and the retina.**

Although the Purkinje cells of the mammalian cerebellum appear remarkably homogeneous with Nissl stains, immunohistochemical methods show that they are diverse with respect to their neurotransmitters, neuropeptides, and enzymes that synthesize neurotransmitters (1–3). γ-Aminobutyric acid (GABA) is the inhibitory neurotransmitter in the majority of Purkinje cells, but others contain taurine and the neuropeptide motilin in mouse, rat, rabbit, and monkey (1–3). Sometimes these substances coexist in single Purkinje cells.

Using double-labeling techniques, Chan-Palay *et al.* (1) found that Purkinje cells are heterogeneous, as some react positively with polyclonal antibodies against glutamate decarboxylase, the GABA-synthesizing enzyme, and therefore have GABA as neurotransmitter (4, 5); others have immunoreactivity toward motilin (1, 6) and a small number have both (1). The significance of that sort of coexistence is not clear, because both GABA and motilin appear to exert inhibitory effects on target cells such as vestibular neurons (7). Apparently some Purkinje cells in the mouse and the rabbit also contain the neurotransmitter taurine, and Purkinje cells lying in the midline of the vermis contain all three substances, presumably as neurotransmitters (3). The remaining 30–40% of all Purkinje cells apparently have neither GABA, motilin, nor taurine in detectable amounts.

In addition to this interesting diversity of Purkinje cells, there are striking parasagittal banding patterns of GABA-, motilin-, or taurine-positive Purkinje cells that alternate with bands of cells negative for these substances. Perhaps this pattern is related to a similar banding of afferent fibers (3). These banding patterns may also be related to longitudinal bands of neurons containing acetylcholinesterase (8), which in the feline cerebellar cortex are connected functionally to the deep cerebellar nuclei (9, 10).

Sagittal banding patterns are also seen particularly in the vermis for the quantitative distribution of 5′-nucleotidase (11, 12) and for the distribution of olivocerebellar afferents and afferents from the lateral reticular nucleus (discussed in ref. 3). Recently Hawkes *et al.* (13) have also reported a monoclonal antibody against adult rat brain that labels Purkinje cells in the adult cerebellum and shows a parasagittal banding pattern of an unknown cell constituent.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Monkey cerebellum is not as well studied as the rodent cerebellum. Sagittal microzones of motilin-containing Purkinje cells in the vermis have been reported (1), but GABA-containing Purkinje cells do not show a banded pattern in the monkey. Most other histochemical or immunocytochemical investigations of zones or bands among the Purkinje cells have been carried out in other mammals. It remains to be seen which observations made in other mammals will apply to the monkey. Functional parasagittal microzones, defined as sets of Purkinje cells that project to a distinct group of target neurons, have been described for the monkey flocculus (14) and may also exist in other parts of the cerebellar cortex of the monkey.

Our monoclonal antibody (mAb) B1, which was produced against rat embryonic forebrain membranes, produces specific and striking immunocytochemical staining of Purkinje cells in the adult monkey cerebellum. The stain shows parasagittal bands of positively stained and of unstained Purkinje cells in the cerebellar hemispheres and patches of stained cells at the edges of the vermal lobes.

MATERIALS AND METHODS

Production of Monoclonal Antibodies. Monoclonal antibodies were produced against 16-day embryonic rat forebrain membranes using an *in vitro* immunization procedure (15, 16). Membrane vesicles were prepared by the method of Edelman and co-workers (17) and monitored by $NaDodSO_4$/polyacrylamide gel electrophoresis and by electron microscopy. For *in vitro* immunization, spleen cells from unimmunized mice were placed in culture and then treated *in vitro* with 100 ng of membrane protein from 16-day embryonic rat forebrain. These spleen cells were fused to P3-X63.Ag8.653 myeloma cells at a ratio of five spleen cells to one myeloma cell with polyethylene glycol (PEG, M_r 4000) and plated with macrophage feeder cells into two 24-well plates in RPMI medium containing 20% fetal calf serum (vol/vol) and hypoxanthine/aminopterin/thymidine (HAT).

After 20 days 41% of the wells showed hybridoma growth, one-third of which were positive in the enzyme-linked immunosorbent assay (ELISA) using the original membrane preparation as antigen. The ELISA for detecting the presence of antibody in cell supernatant is a standard procedure (15, 18).

The hybridoma cells from the first positive wells after fusion were cloned three times by limiting dilution.‡ Cells were frozen as backup stocks at −190°C, grown up, and expanded later to harvest supernatants. Supernatants were concentrated with the Millipore Minitan concentrating apparatus. Ascites fluid was prepared by injecting hybridoma cells

Abbreviations: GABA, γ-aminobutyric acid; mAb, monoclonal antibody.

‡Center for Advanced Training in Cell and Molecular Biology, Catholic University of America, Course on hybridoma/monoclonal antibody production, Boston, Sept. 1983.

into the peritoneal cavity of pristane-primed BALB/c mice. mAbs were characterized by immunocytochemistry.

Histochemical Methods. Tissue was obtained from two *Macaca fascicularis* monkeys who had been deeply anaesthetized with barbiturate and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde (wt/vol) in phosphate buffer, pH 7.4. The brains were removed, blocked into smaller pieces, and immersed in 30% sucrose/phosphate buffer for 36–48 hr. Sections 30 μm thick were cut through two blocks of cerebellum in the sagittal and coronal planes on a freezing microtome and stored in phosphate buffer at 4°C. Immunohistochemical procedures followed the indirect peroxidase-antiperoxidase protocols for light microscopy (19, 20) as described by Hendrickson *et al.* (21). Free-floating sections were incubated with mAb B1 from ascites fluid diluted either 1:10 or 1:100 in P_i/NaCl/0.1% Triton X-100/1% normal goat serum (vol/vol) at 4°C overnight. Following a 1–3 hr rinse in P_i/NaCl, the sections were incubated in goat anti-mouse antiserum (Sternberger/Meyer, Jarrettsville, MD) diluted 1:40 for 2–12 hr at room temperature or for longer periods at 4°C. After a 1–3 hr rinse in P_i/NaCl, the sections were incubated with mouse peroxidase-antiperoxidase (Sternberger/Meyer) diluted 1:100 for 2–12 hr at room temperature or for longer periods at 4°C. Sections were rinsed briefly in P_i/NaCl and then in Tris buffer, pH 7.6, and incubated with diaminobenzidine at 1 mg/ml in Tris buffer containing $\approx$0.01% H_2O_2 for 10–30 min. Sections that reacted were finally rinsed, mounted, dehydrated, and covered with a coverslip for examination by bright-field, phase-contrast, or Nomarski optics microscopy. Control sections were incubated without mAb B1 or with primary antibody from a different hybridoma. Background staining was greatly reduced by preincubation in hydrogen peroxide.

RESULTS AND DISCUSSION

The products from an *in vitro* fusion with 16-day embryonic rat forebrain membranes were distributed into 48 wells. Cells whose supernatant was positive in an ELISA using the immunogen as antigen were cultured and then cloned three times by limiting dilution, using 1 cell per well, then 10 cells per well, and again 1 cell per well dilutions. In each case only those wells in which there was clearly only a single colony were selected. One hybridoma cell line was chosen for further study on the basis of its ability to decorate Purkinje cells; this line produces mAb B1. After the third cloning, cells were injected into pristane-primed mice and ascitic fluid was harvested from several mice.

Tissue Labeling with mAb B1. mAb B1, when visualized by means of the indirect peroxidase-antiperoxidase method, labels neurons in the cerebellum, spinal cord, and motor cortex in a variety of mammals. In addition, some neurons in the somatosensory cortex, vestibular and cochlear nuclei, and the retina are also labeled with B1 (results not shown). In this communication we will concentrate on the cerebellum of the mature macaque monkey.

The somata of monkey cerebellar Purkinje cells form a unicellular layer situated between a more superficial molecular layer and a deeper granular layer. The latter two layers are several times thicker than the Purkinje layer and contain a variety of neuronal types. In Nissl-stained tissue sections the solitary row of relatively large Purkinje cell bodies stands out against a densely stained granular layer and a pale molecular layer like a string of beads and appears homogeneous in cell size, shape, and distribution throughout the cerebellar cortex.

mAb B1 shows intense labeling of many Purkinje cells in the cerebellar cortex. Labeled cells contain a dense brown reaction product that appears to be homogeneously distributed throughout the cytoplasm (Figs. 1 and 2) and is excluded from the nucleus. The large, brown reactive Purkinje cell somata are easily detected at low magnification against a virtually colorless background (Figs. 1 and 2). In some

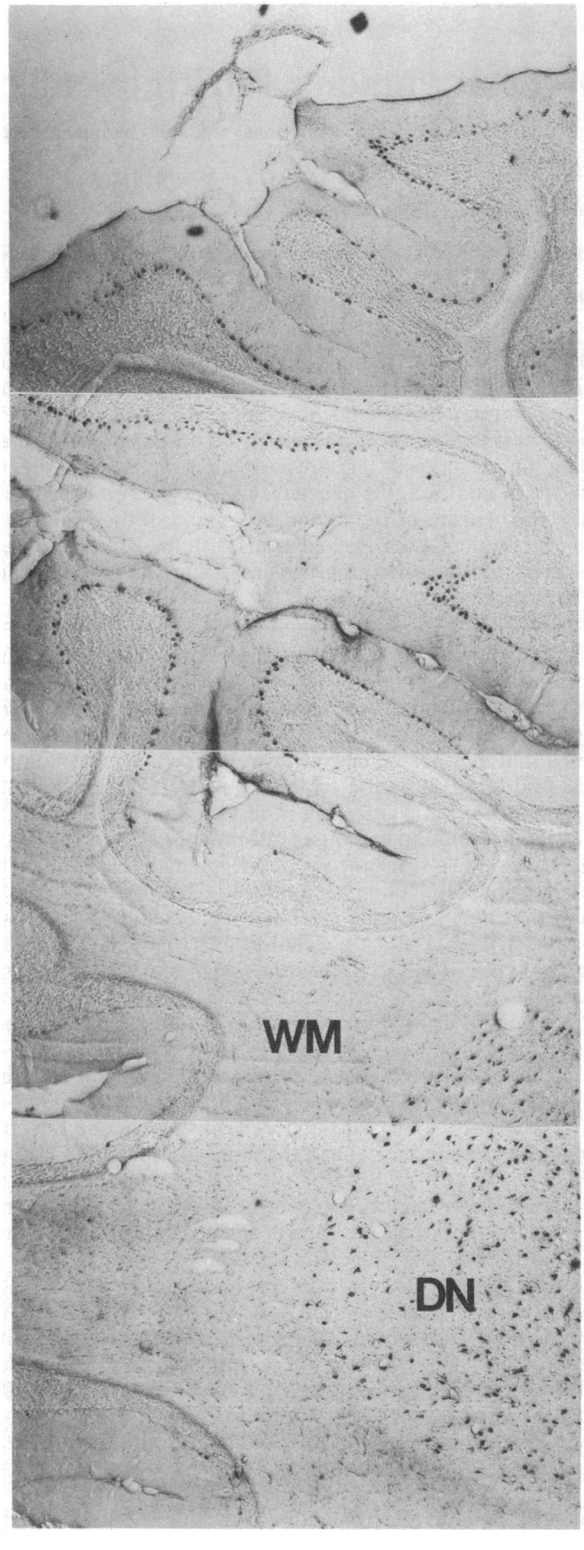

FIG. 1. Sagittal section of adult monkey cerebellum showing B1-containing neurons labeled with the peroxidase-antiperoxidase method; no counterstain. Bright field illumination. Photomontage through intermediate part of hemisphere, dorsal surface at the top. Part of the deep cerebellar nuclei is shown (DN) opposite the white matter (WM). Stained Purkinje cells form patches interrupted by patches of unlabeled Purkinje cells. (×5.4.)

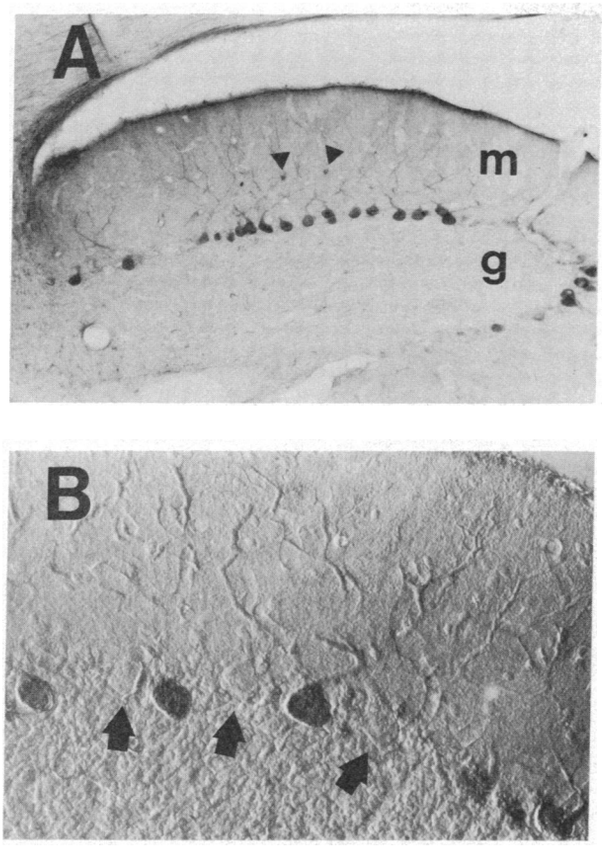

FIG. 2. The same sagittal section as Fig. 1, stained with mAb B1 to show labeled and unlabeled Purkinje cells and their dendritic trees. (*A*) In addition to the Purkinje cells and their dendritic trees, a few small cells in the molecular layer (m) are also labeled (arrowheads). The granular layer (g) is unlabeled. (Bright-field micrograph; ×12.) (*B*) Another area of the same section. Solid arrows point out unlabeled Purkinje cells next to labeled Purkinje cells. (Nomarski optics; ×23.)

preparations, the Purkinje dendrites also stain well, especially in their proximal branches (Fig. 2), which can be followed into the molecular layer but are eventually lost as the labeling fades to background levels at the upper one-third of the layer.

In addition to the Purkinje cells of the cerebellar cortex, small scattered B1-containing neurons are also found in the deep half of the molecular layer. Occasionally other poorly defined constituents of the Purkinje cell layer are also labeled (see Fig. 5). These may be portions of basket cells or glial cells, that appear to be less intensely labeled than the Purkinje cells themselves.

Neurons of the four deep cerebellar nuclei are also intensely labeled by mAb B1 (see Fig. 1). Their cytoplasm is as strongly immunoreactive as that of the Purkinje cells. In addition some weak immunolabeling is found in the neuropil. Unlike the Purkinje cells, virtually all neurons of the deep nuclei contain B1 antigen.

Control sections were run in parallel but without primary antibody in the first incubation or with ascites fluid from different hybridoma cell lines, whose antibodies did not bind to cerebellar Purkinje cells. Control sections were negative throughout the cerebellar cortex and the deep nuclei.

The most striking aspect of the Purkinje cell labeling pattern in the adult monkey is its patchiness or banding pattern seen in both coronal and sagittal sections (Figs. 1, 3, and 4). The distribution of labeled Purkinje cells in sections of the hemispheres and the vermis shows clusters of labeled cells intermixed with clusters of unlabeled cells in strings consisting of 5–20 labeled (or unlabeled) neurons in a row.

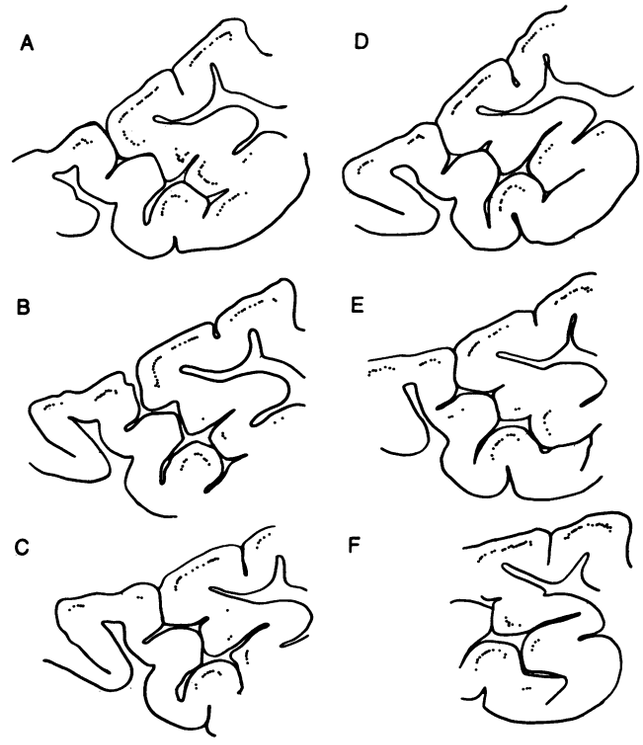

FIG. 3. Camera lucida drawings of coronal sections of adult monkey cerebellum stained with mAb B1. The sequence of six consecutive sections (*A–F*) covers a distance of approximately 0.5 mm in a caudal to rostral direction. Only mAb B1-containing Purkinje cells are shown, represented by black dots. The obvious gaps left in the Purkinje cell layers are occupied by B1-lacking cells or faintly labeled cells. The clusters of B1-containing cells are almost superimposable in succeeding sections. (×6.)

Preliminary results from acetylcholinesterase detection in adjacent sections indicate that the striped distribution pattern of this enzyme as described in the cat by Marani and Voogd (22) and by Brown and Graybiel (10) is also found in the vermis of the monkey cerebellum. However, the pattern of B1-binding Purkinje cells does not appear to coincide with the distribution of acetylcholinesterase-containing cells.

We have examined the clusters of Purkinje cells that either do or do not contain the B1 antigen in sequential coronal sections in an attempt to discover the B1 pattern of distribution. B1-containing and B1-lacking cell clusters are almost

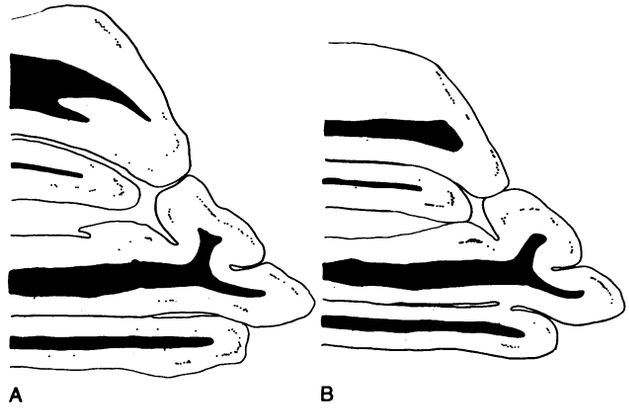

FIG. 4. Coronal sections through the vermis of an adult monkey cerebellum labeled with mAb B1; only B1-containing Purkinje cells are shown, represented by black dots. The section shown in *A* is located 270 μm caudally to that shown in *B*. (×4.6.)

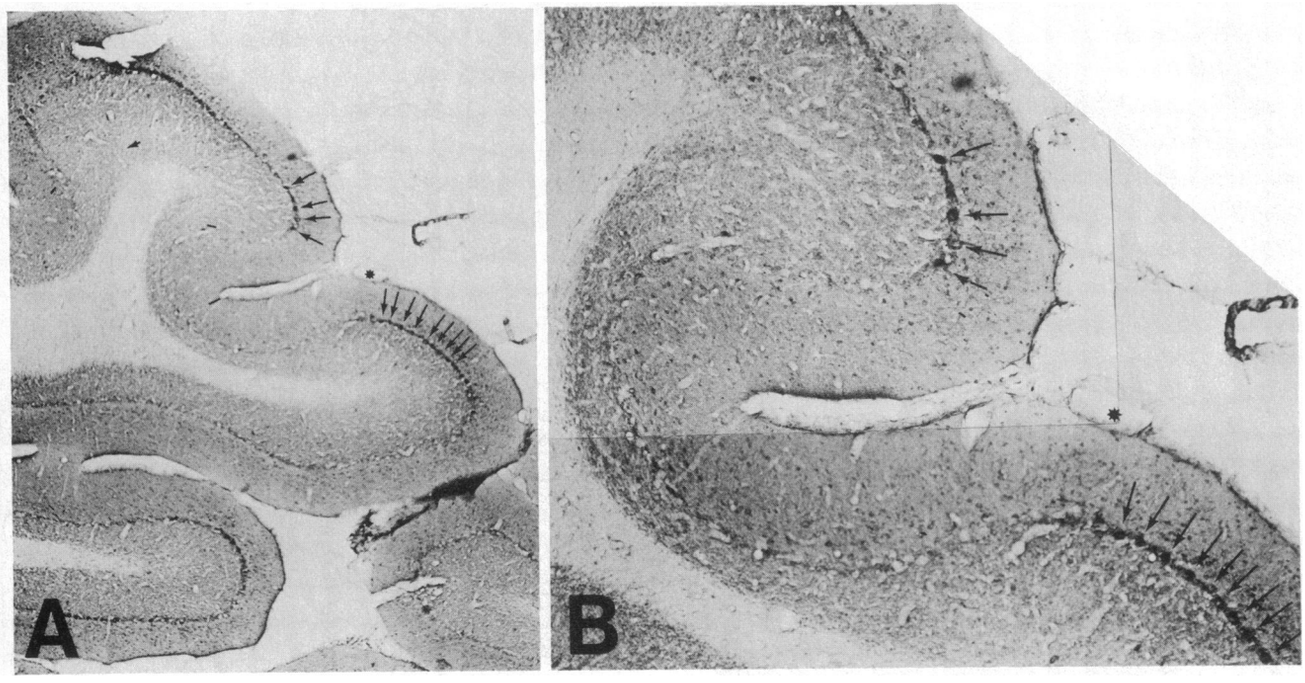

FIG. 5. A portion of the coronal section of the adult vermis drawn in outline in Fig. 4*B*. Arrows point to B1-containing Purkinje cells; the asterisk marks a blood vessel in both *A* and *B*. (*A*, ×25; *B*, ×60.)

precisely superimposed over the 0.5 mm in the caudal-rostral direction that was examined (Fig. 3). Therefore, the patches or bands of B1-antigen-containing cells are at least 0.5 mm long. It remains to be determined how far the patches or bands extend in this direction, although it is our impression that a given patch may end or begin within 0.5 mm.

The pattern of B1-containing Purkinje cells in the monkey vermis is particularly interesting (Figs. 4 and 5). These cells are clustered at the edges of the lobes, whereas the center of each lobe is occupied almost exclusively by cells without B1 antigen.

Finally, it is noteworthy that other structures contain neurons which label with mAb B1. These include large and small infragranular neurons in the motor cortex and large spinal motor neurons of the ventral horn. It should be emphasized, however, that sensory neurons at various stations are also decorated by mAb B1, such as the sensory spinal neurons of the dorsal horn, some neurons in the somatosensory cortex, some cells in the vestibular and cochlear nuclei, and the retina.

The important finding here described is that mAb B1 specifically recognizes an antigen in Purkinje cells and a restricted number of other neuronal cells. In the adult monkey the distribution of this antigen among the Purkinje cells is heterogeneous with B1-containing Purkinje cells arranged in patches or bands running in a rostrocaudal direction.

The bands of Purkinje cells containing B1 antigen seem to be different from the rostrocaudal bands reported by Chan-Palay *et al.* (1) for motilin-containing and glutamate decarboxylase-containing Purkinje cells in the adult rat brain. Our bands are much broader (5–20 Purkinje cells wide), and we do not see the microzonation of labeled Purkinje cells in the central vermis reported by these authors. In addition, the sagittal microbands of taurine-containing Purkinje cells (3) reported by the same laboratory also look quite unlike the banding pattern which we describe here. There are, however, two reasons for caution in making these comparisons. First, we do not yet know the identity of our antigen and, second, the discrepancy may result from a species difference between our findings in the adult monkey and previous studies in the adult rat. However, in parallel studies with the adult rat (unpublished results) we have found a B1 distribution pattern among the Purkinje cells which resembles our findings in the monkey but does not resemble the banding pattern reported by Chan-Palay *et al.* (1, 3).

The B1 antigen should be among the proteins or glycoproteins of the membranes from 16-day embryonic rat forebrains, since these membranes were the original immunogen. We must point out that we did use a crude membrane preparation and, therefore, might well have included contaminating cytoplasmic proteins. The strength of the mAb approach is the ease of identifying antigens in tissues and isolating specific antigens from mixtures. The weakness of the method lies in the possibility that two different protein antigens may have the same epitope recognized by a single mAb; only further analysis can sort out these possibilities. Furthermore, it remains to be seen whether the B1 antigen(s) is system specific, such as those described for the limbic (23) and visual systems (24). Further investigation with B1 in rat embryos might also give an indication of the cell lineages involved in the differentiation of Purkinje cells.

We would like to thank Drs. Ann M. Graybiel and Walle J. Nauta for their help and advice, and Ms. Helen Moore for important discussions. This work was supported by Whitaker College, Massachusetts Institute of Technology, by the March of Dimes Foundation, and by the Whitaker Health Sciences Fund. C.L.C. holds a Public Health Service postdoctoral fellowship and J.M.G. a predoctoral fellowship from the National Science Foundation.

1. Chan-Palay, V., Nilaver, G., Palay, S. L., Beinfeld, M. C., Zimmerman, E. A., Wu, J.-Y. & O'Donohue, T. L. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7787–7791.
2. Chan-Palay, V., Lin, C.-T., Palay, S. L., Yamamoto, M. & Wu, J.-Y. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2695–2699.
3. Chan-Palay, V., Palay, S. L. & Wu, J.-Y. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 4221–4225.
4. Wood, J. G., McLaughlin, B. J. & Vaughn, J. E. (1976) in

GABA in Nervous System Function, eds. Roberts, E., Chase, T. N. & Tower, D. B. (Raven, New York), pp. 133–148.
5. Obata, K., Ito, M., Ochi, R. & Sato, N. (1967) *Brain Res.* **4**, 43–57.
6. Nilaver, G., Defendini, R., Zimmerman, E. A., Beinfeld, M. C. & O'Donohue, T. L. (1982) *Nature (London)* **295**, 597–598.
7. Chan-Palay, V., Ito, M., Tongroach, P., Sakurai, M. & Palay, S. L. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3355–3359.
8. Marani, E. (1982) Dissertation (Univ. of Leiden, Leiden, Netherlands).
9. Brown, B. L. & Graybiel, A. M. (1983) *Soc. Neurosci. Abstr.* **9**, 1091.
10. Brown, B. L. & Graybiel, A. M. (1983) *Anat. Rec.* **205**, 25A (abstr.).
11. Scott, T. G. (1964) *J. Comp. Neurol.* **122**, 1–7.
12. Marani, E. & Voogd, J. (1973) *Acta Morphol. Neerl. Scand.* **11**, 365.
13. Hawkes, R., Leclerc, N. & Colonnier, M. (1984) *Soc. Neurosci. Abstr.* **10**, 44.
14. Ito, M. (1984) *The Cerebellum and Neural Control* (Raven, New York), p. 192.
15. Pardue, R. L., Brady, R. C., Perry, G. W. & Dedman, J. R. (1983) *J. Cell Biol.* **96**, 1149–1154.
16. Matthew, W. D. & Patterson, P. H. (1983) *Cold Spring Harbor Symp. Quant. Biol.* **48**, 625–631.
17. Hoffman, S., Sorkin, B. C., White, P. C., Brackenbury, R., Mailhammer, R., Rutishauser, U., Cunningham, B. M. & Edelman, G. M. (1982) *J. Biol. Chem.* **257**, 7720–7729.
18. European Molecular Biology Organization (1980) *Hybridoma Techniques* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
19. Sternberger, L. A. (1979) *Immunocytochemistry* (Wiley, New York), pp. 104–169.
20. Vaughn, J. E., Barber, R. P., Ribak, C. E. & Houser, C. R. (1981) *Current Trends in Morphology Techniques*, ed. Johnson, J. E., Jr. (CRC, Boca Raton, FL), pp. 33–70.
21. Hendrickson, A. E., Ogren, M. P., Vaughn, J. E., Barber, R. P. & Wu, J.-Y. (1983) *J. Neurosci.* **3**, 1245–1262.
22. Marani, E. & Voogd, J. (1977) *J. Anat.* **124**, 335–345.
23. Levitt, P. (1984) *Science* **233**, 294–301.
24. Hockfield, S., McKay, R. D., Hendry, S. H. C. & Jones, E. G. (1983) *Cold Spring Harbor Symp. Quant. Biol.* **48**, 877–889.