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Sagittal cerebellar microbands of taurine neurons: Immunocytochemical demonstration by using antibodies against the taurine-synthesizing enzyme cysteine sulfinic acid decarboxylase

(Purkinje cell/interneurons/vermis/flocculonodular)

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ABSTRACT Taurine neurons in the cerebellum of rabbit, rat, and mouse were localized at the light microscope level by using polyclonal antibodies against cysteine sulfinic acid decarboxylase (CSADCase; EC 4.1.1.29), the enzyme responsible for the conversion of cysteine sulfinic acid to hypotaurine and of cysteic acid to taurine. The indirect peroxidase-antiperoxidase method was used on Vibratome sections and on serial sections of paraffin-embedded tissue. Intensification of CSADCase immunoreactivity was achieved by pretreatment of the animal with L-cysteine or L-cysteic acid intravenously 1–2 hr prior to perfusion. A combination of L-cysteic acid and demecolcine, which retards axoplasmic flow, was most effective in maximizing CSADCase immunoreactivity. Although these treatments intensified immunoreactivity in neurons, no more cells were reactive than in untreated controls. L-Glutamic acid did not increase CSADCase immunoreactivity but did increase immunoreactivity with antibodies against L-glutamic acid decarboxylase (GAD; EC 4.1.1.15), the synthetic enzyme for γ-aminobutyric acid. Specificity was established by negative results obtained with various control incubations including the use of CSADCase antiserum preabsorbed with the antigen. Taurine neurons of the cerebellar cortex are arranged in sagittal microbands, defined by intensely CSADCase-reactive Purkinje neurons and their axons and dendrites, together with stellate, basket, and Golgi cells and their processes. In the vermis there is a narrow midline band, flanked laterally by three wider bands on either side, each separated from the next by an unreactive zone. Although the zonal borders are sharp, the interzonal areas contain some CSADCase-immunoreactive axons but no cell bodies. The seven vermal bands are best observed in the anterior lobe. Others exist in the lateral hemispheres. The paraflocculus and flocculus contain numerous intensely immunoreactive neurons, and banding is difficult to discern. Lobule X of the vermis is also heavily endowed with taurine neurons. Numerous large and medium-sized deep cerebellar and vestibular nuclei are also immunoreactive. These observations indicate that cerebellar neurons are chemically heterogeneous but that neurons of similar chemical signature in the cerebellar cortex are organized into sagittal microbands. This corroborates our earlier evidence that Purkinje cells containing motilin and those containing both motilin and γ-aminobutyric acid are also arranged in vermal sagittal microbands. The midline vermal band contains Purkinje neurons with multiple neuroactive substances—taurine, γ-aminobutyric acid, and motilin. It remains to be determined how this chemical zonation in the cerebellar cortex relates to the banded afferent innervation from spinal, vestibular, reticular, and olivary sources.

Taurine has been suggested as a neurotransmitter in the mammalian central nervous system (1) and is inhibitory in the cerebellum (2). L-Cysteine sulfinic acid decarboxylase (CSADCase; EC 4.1.1.29) is the enzyme responsible for taurine synthesis in brain through the decarboxylation of cysteine sulfinic acid to hypotaurine with subsequent oxidation to taurine or, alternatively, by the decarboxylation of cysteic acid to taurine (3). CSADCase has been successfully separated from L-glutamic acid decarboxylase (GAD; EC 4.1.1.15) from bovine brain and purified to homogeneity (4). GAD is responsible for the synthesis of γ-aminobutyric acid (GABA). Antibodies raised against each substance show no crossreactivity (4). Thus, taurine and GABA neurons are readily differentiated by means of immunochemical techniques, and we have recently reported that taurine neurons exist in the cerebellum (5).

The cerebellar cortex is organized on an orthogonal plan (6), and the entire cerebellum has been divided into a number of narrow sagittal bands according to detailed study of the cerebellar white matter (7). Physiological experiments also indicate that the spinal and olivary afferents to the cortex are arranged in parasagittal zones (8). Neuroanatomical investigations show that olivo-cerebellar afferents follow a strict parasaggital pattern in the cortex (9–15) as do the afferents from the lateral reticular nucleus (10). Histochemical studies demonstrate that both acetylcholinesterase (16) and 5′-nucleotidase (17, 18) are distributed in a banded pattern in the cerebellar cortex. Both enzymes appear to be associated with Purkinje cell dendrites. Our recent observations (19) disclosed a chemically specified sagittal microzonation which is defined by the distribution of Purkinje cells containing motilin alone or motilin together with GABA. These observations lead to the inescapable conclusion that the cerebellum has an intrinsic chemical organization, which is based upon a sagittal banding scheme.

This communication describes a set of cerebellar sagittal bands on the basis of immunocytochemistry with CSADCase antibodies. A use of pharmacological pretreatment with specific enzyme substrates is also presented.

METHODS AND MATERIALS

Two methods were used for light microscope immunocytochemistry on paraffin sections and Vibratome sections. Young adult albino rabbits (1.5–2 kg), rats (200–300 g), and inbred Swiss albino mice (30 g, Charles River Breeding Laboratories)

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Abbreviations: PAP, peroxidase-antiperoxidase; CSADCase, cysteine sulfinic acid decarboxylase; GAD, glutamic acid decarboxylase; GABA, γ-aminobutyric acid.

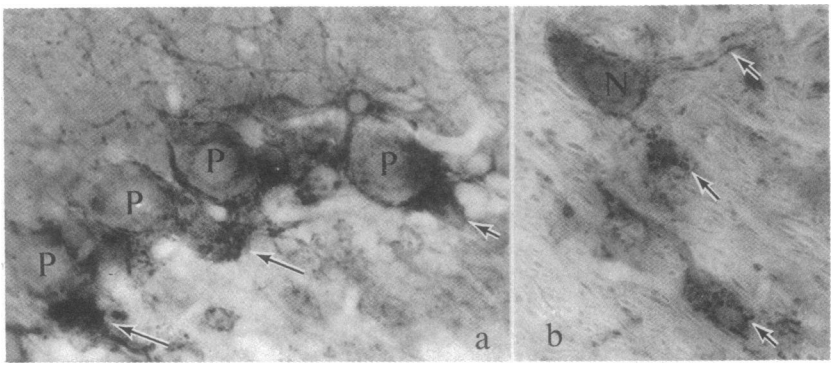

FIG. 1. Comparative sections (rat) with GAD immunoreactivity. (*a*) Purkinje neurons (P) are lightly "reactive"; the predominant reaction is in the axons of basket cells in the pinceau (arrow). (*b*) Purkinje axons (arrows) on the surfaces of dendrites and somata of neurons in the deep cerebellar nuclei. (×540.)

were used. Animals were divided into six groups: (*i*) normal untreated controls; (*ii*) treated with demecolcine (Sigma), 1 mg/kg intraperitoneally 16 hr before fixation; (*iii*) treated with L-glutamate, 2 ng/kg intravenously 1–2 hr before fixation; (*iv*) treated with L-cysteine, 1.3 ng/kg intravenously 1–2 hr before fixation; (*v*) treated with L-cysteic acid, 2 ng/kg intravenously 1–2 hr before fixation; (*vi*) treated with harmaline, 5 mg/kg intravenously (20), 1 hr before fixation. Anesthesia before perfusion was with pentobarbital or urethane for rabbits, 35% chloral hydrate for rats, and with ether for mice. Animals for paraffin embedding were perfused through the heart with calcium-free Tyrode's solution and then with Bouin's fixative at 4°C (formaldehyde/picric acid/glacial acetic acid). Serial sections were cut at 10 μm for immunocytochemistry studies. For Vibratome sections, the brains were perfused with Tyrode's solution followed by 4% formaldehyde/0.12 M phosphate, pH 6.5, and then 4% formaldehyde/0.12 M phosphate buffer, pH 11.0, at 4°C. The brains were then freed from the skulls, trimmed, and left overnight in 4% phosphate-buffered formaldehyde (pH 7.4). Serial sections were cut at 20 μm and the loose sections were subjected to immunocytochemical reactions. For the immunocytochemical procedures, 0.1 M Tris pH 7.6 buffer was used throughout.

Similar procedures were used for paraffin and Vibratome sections with the exception of antibody concentrations and duration. CSADCase antibodies were applied to paraffin sections at 1:100 dilutions at 4°C overnight and to Vibratome sections at 1:1,000 dilutions at 37°C for 1.5 hr. All antibodies and conjugates were diluted with 0.1 M Tris pH 7.6 buffer containing 0.01% saponin and 1% normal goat serum. The sections were incubated according to the following sequence with extensive buffer rinses between steps: 25 mM hydroxylamine to bind excess aldehyde, 10% (vol/vol) normal goat serum (to bind nonspecific sites) plus 0.1% saponin (to aid antibody penetration), primary antibody, peroxidase-conjugated goat anti-rabbit IgG (1:100), rabbit peroxidase-antiperoxidase (PAP) (1:100), diaminobenzidine (0.022%) alone for 5 min and then together with 0.003% hydrogen peroxide for 6 min. Loose Vibratome sections were mounted on chrome alum/gelatin covered slides. Control treatments included incubation of sections in CSADCase antibodies maximally preabsorbed overnight with the antigen CSADCase, rabbit preimmune serum, antiserum against ovalbumin, incubation without primary antibodies or conjugates. Comparison material was obtained from sections incubated in polyclonal and monoclonal anti-GAD. All Vibratome and paraffin sections were rapidly dehydrated and coverslipped with Permount. Sections were viewed with conventional or Nomarski optics. Serial sections through the cerebellum were mapped to determine the locations and numbers of individual CSADCase-immunoreactive neurons throughout the cerebellum.

RESULTS

Substrate Enhancement. CSADCase immunoreactivity in cerebellar neurons was readily detectable in both paraffin and Vibratome sections in all species examined, and such sites were confirmed by comparison with control sections. A comparative study was done to determine how effective the various pre-

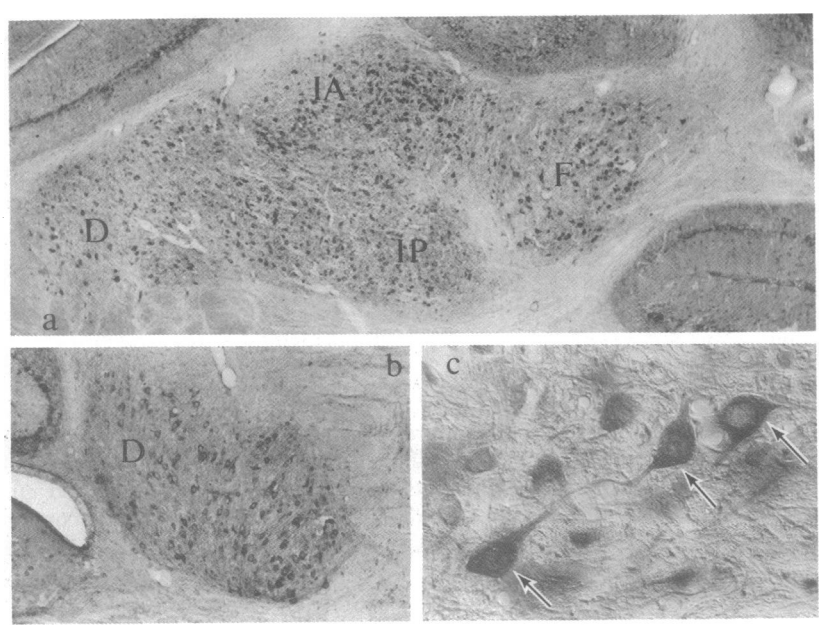

FIG. 2. Taurine neurons of the mouse fastigial (F), interpositus anterior (IA), interpositus posterior (IP), and dentate nuclei (D) shown in coronal (*a*) and sagittal (*b*) sections. (*c*) Medium-sized and large taurine neurons (arrows) of the dentate nucleus in mouse cerebellum. (*a*, ×30; *b*, ×40; *c*, ×340.)

treatments were in increasing specific CSADCase immunoreactivity above that in normal, untreated animals. These specimens indicated that cysteic acid and L-cysteine (the substrates for CSADCase) or their analogs were more effective at increasing staining intensity than were the other treatments tried. Harmaline, which increases cerebellar activity in a rhythmic tremor, increased specific immunoreactivity in CSADCase-positive cells but generally also increased the background coloration, possibly by increasing neural activity. Demecolcine increased immunoreactive levels somewhat but less than the specific substrates, presumably by decreasing axoplasmic transport in nerve fibers and thereby increasing detectable enzyme levels in cell bodies. L-Glutamic acid made no detectable difference in staining intensity, possibly because it is not a substrate for CSADCase. The highest intensification of CSADCase immunoreactivity was obtained by a combined regimen of specific substrate administration (cysteic acid or L-cysteine) together with demecolcine pretreatment. The enhancement in immunoreactivity was observed only in the intensity of the detectable reaction. Comparative counts of Purkinje cell bodies stained in the flocculus indicated that the numbers of immunoreactive cells did not change.

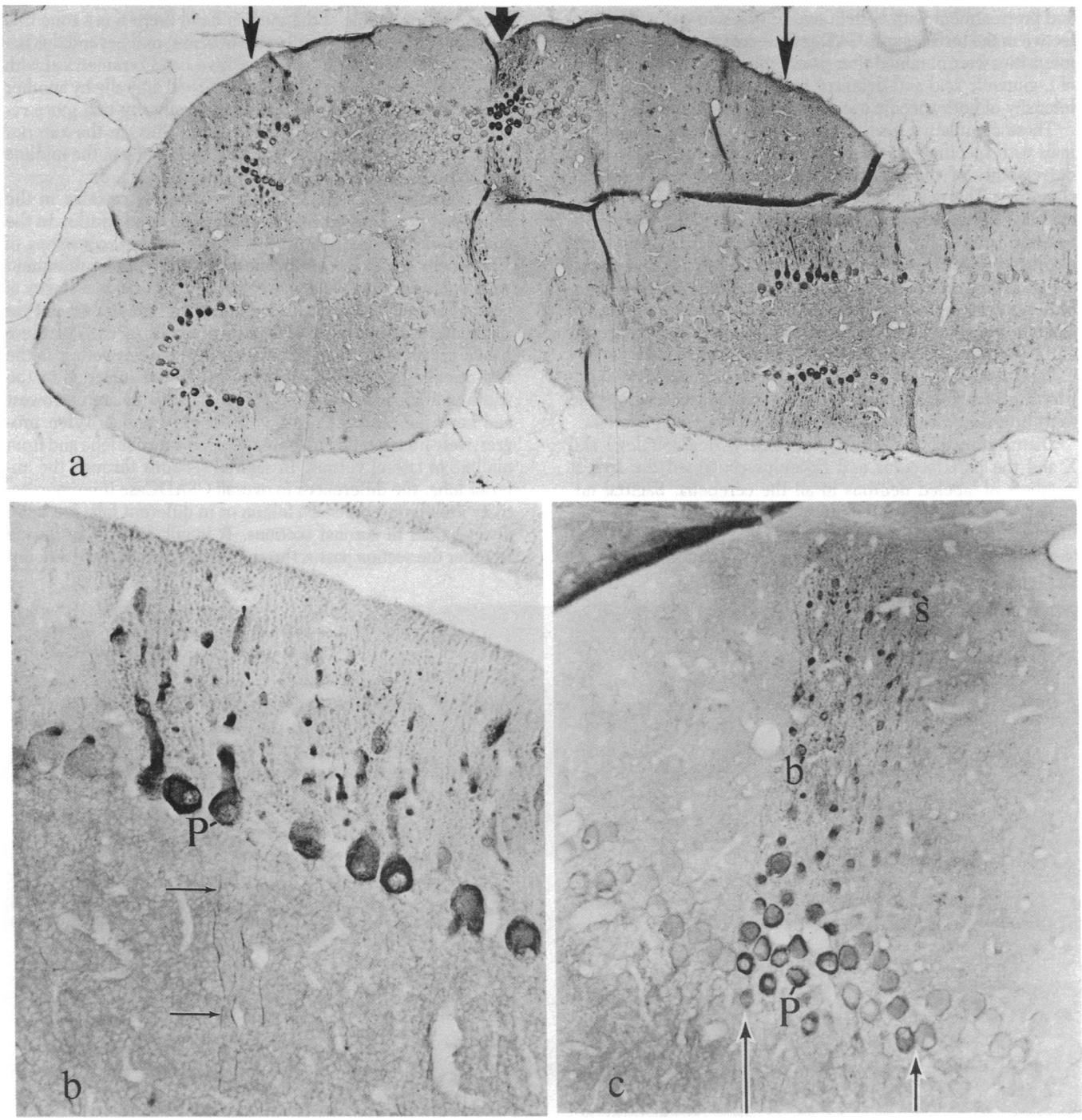

FIG. 3. (*a*) Coronal section through lobule IV in the rabbit vermis showing the median sagittal band (thick arrow) flanked on either side by a free interband and then by a second set of wider bands (thin arrows). (*b*) Higher magnification of two other bands in coronal section showing somata, axons (arrows), and dendrites of Purkinje cells (P) within a band. (*c*) Purkinje cells (P) in a band with stellate (s) and basket neurons (b). The edges of the band are sharp (arrows). (Anti-CSADCase; *a*, ×55; *b*, ×300; *c*, ×200.)

Control sections incubated in antibody preabsorbed with CSADCase were not reactive nor were sections from which conjugates were omitted. Sections treated with GAD antibodies displayed intensely reactive Purkinje, stellate, and basket cells in the cortex and numerous axonal terminals on cells of the deep cerebellar nuclei (Fig. 1). The distribution and characteristics of immunoreactivity in these cells were different from the immunoreactivity associated with anti-CSADCase (Fig. 2; see refs. 5, 19, and 21). Substrate enhancement *in vivo* prior to fixation and anti-GAD reaction in sections resulted in intense immunoreactivity. Treatment of the animals with L-glutamic acid was more effective in this respect than harmaline or demecolcine, and pretreatment with cysteic acid or cysteine was still less effective in the tests for anti-GAD immunoreactivity. The highest intensities were obtained after pretreatment with a combination of L-glutamic acid and demecolcine. Harmaline increased the intensity of both specific staining and the background.

These cumulative results indicate that pretreatment of animals with specific enzyme substrates effectively enhances the detectability of the corresponding enzyme by immunocytochemistry. This manipulation provides an important tool for facilitating the cytochemical application of antibodies against enzymes related to the synthesis of neuroactive substances without losing specificity. The explanations for this enhancement are probably the formation of stable enzyme–substrate complexes, which protect the enzyme from inactivation during the fixation procedure, and conformational changes, which result in the exposure of more antigenic determinants to the antibody molecule and thus improve detectability by immunocytochemistry. Enzyme induction is a less likely possibility because of the short interval between substrate administration and fixation.

Distribution of CSADCase Neurons. Vermal lobules I and X and the paraflocculus and flocculus contained the largest numbers of labeled neurons in all the cerebella. Sagittal microbands of CSADCase-immunoreactive neurons occurred in the cerebella of all species examined. The molecular layer, and to a lesser extent the granular layer, throughout all lobules of the anterior lobe contained symmetrically disposed CSADCase-positive sagittal bands alternating with negative zones (Figs. 3 and 4). The white matter was not stained. In coronal sections, a sharp, narrow band appeared precisely in the midline (Fig. 3*a*) and contained intensely reactive Purkinje cell bodies, 12–16 across the band, with their dendrites extending into the molecular layer as well-defined vertical columns within the band. Intensely stained basket and stellate cells participated in the band. Their processes, particularly the axons of the former, formed a conspicuous network in the lower third of the molecular layer (Fig. 4). The axons of Purkinje cells coursed through the granule cell layer (Fig. 3*b*), continuing the band into this layer. On either side of the median band there was a zone free from CSADCase-immunoreactive neurons, and beyond this laterally was another CSADCase-positive band symmetrical with one on the opposite side. This was followed laterally by another nonreactive band and, still farther laterally, by two more reactive bands separated by a clear zone. Thus, in the anterior lobe, there is a total of seven reactive bands, one in the midline and three pairs of bands on either side of it (Fig. 5).

The somata of Purkinje cells are intensely reactive in the bands and provide a useful measure of the band widths. In the adult animal the midline band is usually narrow, consisting of 12–16 cells across its width; the next lateral and subsequent pairs contain almost 50% more (18–24). (In the late stages of cerebellar development, when the Purkinje cell layer is not yet a monolayer, there are twice as many cells in each of these bands; presumably, subsequent growth of the cerebellum in the rostrocaudal direction decreases the number included in the width of a band in coronal sections.) The bands are narrowest and have the sharpest margins in lobule I and broaden progressively from lobule I to lobule V in the anterior lobe and from midline to lateral vermis. In sagittal sections through the anterior lobe, the differences in overall CSADCase immunoreactivity in different parts of a folium or in different folia are more distinct than in coronal sections. It is not difficult to decide whether the section passes through a band or not by the amount

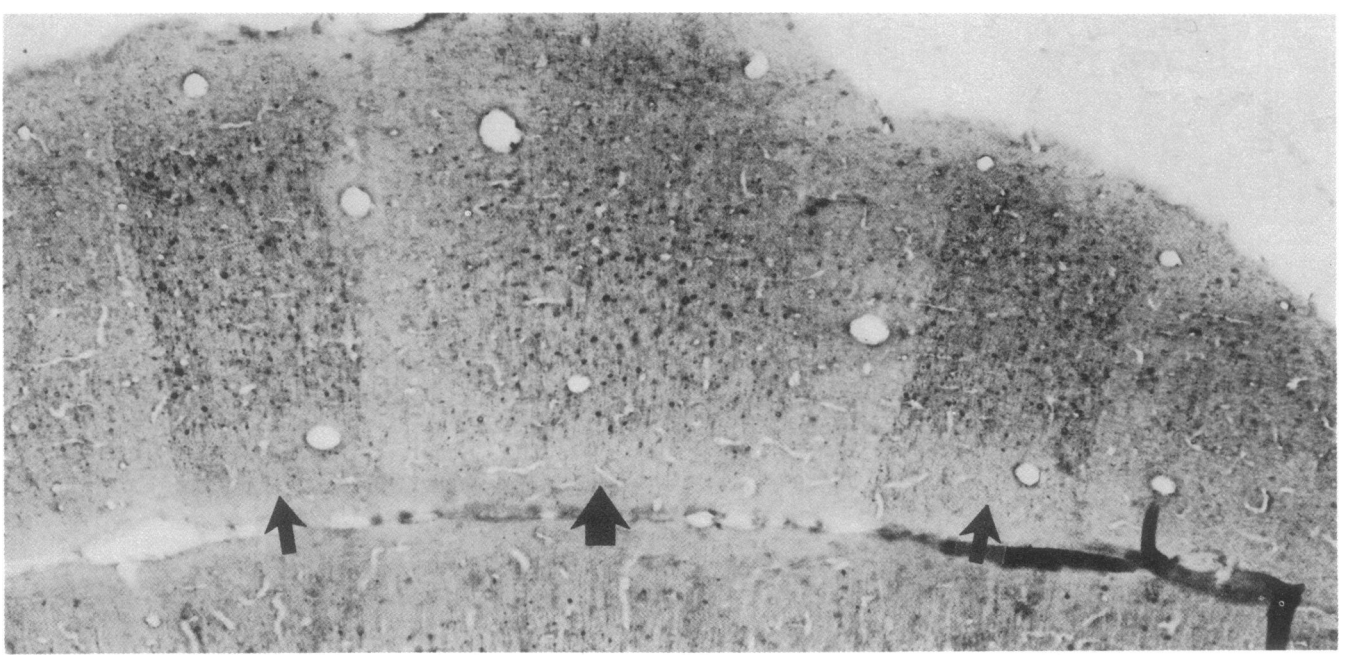

FIG. 4. Grazing section in the coronal plane through the lower third of the molecular layer in lobules V a and b in the rabbit vermis. The median band (large arrow) and lateral bands (smaller arrows) are separated by interband regions containing only a few immunoreactive axons. Within the bands the major CSADCase-positive components are a grid of Purkinje cell dendrites in transverse section, numerous stellate and basket cells, and their reactive processes. (Anti-CSADCase; ×200.)

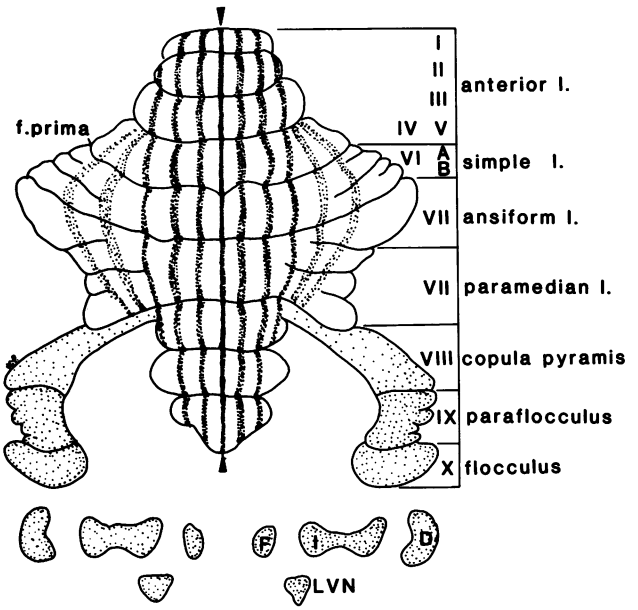

FIG. 5. Schematic summary of the CSADCase-positive sagittal microzones or bands in mouse cerebellum (foliation scheme modified from ref. 22). The bands are clearest in the anterior lobe and the vermis, less sharply defined in the hemispheres (dense stipple), and most difficult to discern in the paraflocculus and flocculus (light stipple), because of intense CSADCase reactivity in most Purkinje cells. The dentate (D), interpositus (I), fastigial (F), and lateral vestibular nuclei (LVN) contain numerous taurine cells.

of staining in neurons in the successive folia.

In the posterior lobe of the vermis, the bands are present but are less distinct, and bands in the hemispheres are difficult to distinguish. In the hemispheres of lobules VI and VII, at least two bands can be delineated. They vary in width from 8 to 18 Purkinje cells in coronal section and conform to the complex lobulations of the folia. The paraflocculus and flocculus contain the highest proportion of intensely CSADCase-positive neurons, and reaction-free zones are difficult to discern (Fig. 5, light stipple). Estimates indicate that at least 60–65% of Purkinje cells are CSADCase positive. The deep cerebellar nuclei and lateral vestibular nucleus (Fig. 2) contain numerous medium-sized to large neurons with CSADCase immunoreactivity. It was not possible to discern a banding pattern in these nuclei.

DISCUSSION

The present demonstration that the taurine neurons of the cerebellar cortex are organized into precise sagittal microzones corroborates earlier observations that motilin- and GABA-containing Purkinje cells are similarly arranged (19). In fact, the motilin/GABA Purkinje cell band in the midline of the cerebellum overlaps with the midline CSADCase-positive band. Purkinje cells lying in the true midline of the vermal cerebellar cortex (approximately four to eight cells wide) contain coexistent taurine, motilin, and GABA. Thus, these Purkinje cells have multiple chemical messengers. With such a variety of different neuroactive substances coexisting in Purkinje cells and with the sagittal organization precisely defined, it becomes imperative to determine how this intrinsic chemistry of the cerebellar cortex relates to the innervation patterns defined by afferent spinal, vestibular, reticular, olivary, or pontine fibers. The distribution of Purkinje cells with different chemical signatures must have a functional correlate because both afferent fiber systems and chemical characteristics are arranged in parallel, longitudinal, or parasagittal strips or compartments. The compartments for each feature, chemical or neuroanatomical, however, are not coincident in size, location, composition, or pattern but overlap and interdigitate in a complex and as yet unanalyzed manner.

The observations accumulated in the present and previous studies (2, 5, 19) invalidate the claim that all Purkinje cells are uniformly GAD immunoreactive (23) and GABA-ergic. The ordered distribution of Purkinje cells with different chemical signatures throughout the cerebellar cortex indicates that they, and probably other cerebellar cells, are arranged in a mosaic of interlocking specificities, reflecting their topographical and functional roles.

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