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Chemical heterogeneity in cerebellar Purkinje cells: Existence and coexistence of glutamic acid decarboxylase-like and motilin-like immunoreactivities

(morphology/sagittal microzones/motilin/glutamate dehydrogenase)

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ABSTRACT **Purkinje neurons of the cerebellar cortex form a chemically and morphologically heterogeneous population containing some members that have γ-aminobutyric acid (GABA), others that have immunoreactivity for motilin, and a small number that have both. The remaining 30–40% of all Purkinje cells have neither of these two neuroactive substances, leaving possibilities for other transmitter candidates. The evidence was compiled from double-staining immunocytochemical procedures performed on single sections of the cerebellum and brain stem in rat, mouse, and monkey. Two polyclonal antibodies were applied in succession, one directed against the midregion and COOH terminus of the 22-amino acid polypeptide motilin and the other against glutamic acid decarboxylase (glutamate decarboxylase; L-glutamate 1-carboxy-lyase, EC 4.1.1.15), the rate-limiting enzyme in the synthesis of the neurotransmitter GABA. The staining combinations employed the immunoperoxidase method, with different chromogens for distinguishing the motilin-like immunoreactivity from glutamic acid decarboxylase immunoreactivity by different colors, or the immunoperoxidase method for one antiserum and immunofluorescence for the other. The locations of both motilin and GABA cell types were mapped. The recognition of motilin in Purkinje cells calls for experimental definition of the role of this substance in the cerebellum and for reevaluation of the roles of Purkinje cells and of GABA in cerebellar function. The significant motilin representation in the flocculus, paraflocculus, and vermis suggests that it may be the Purkinje cell mediative chemical in the vestibular parts of the cerebellum. However, the presence of GABA as well in the same regions indicates that the chemical preference may be at least bimodal.**

A considerable literature supports the identification of γ-aminobutyric acid (GABA) as a major inhibitory transmitter in the cerebellum. GABA has been localized by autoradiography with uptake of tritiated GABA (1) or binding of tritiated muscimol for muscimol–GABA receptor sites (2, 3), and by immunocytochemistry with antibodies directed against glutamic acid decarboxylase (GAD; glutamate decarboxylase; L-glutamate 1-carboxy-lyase, EC 4.1.1.15), the rate-limiting enzyme for the synthesis of GABA from glutamic acid (4–8), and with antibodies against γ-aminobutyric acid transaminase, the enzyme that degrades GABA (4, 9). Of the cerebellar neurons, a majority of the smaller interneurons—e.g., stellate, basket, and Golgi cells—are labeled consistently by these cytochemical methods for localization of GABA. The Purkinje cells, however, have remained enigmatic. Although a considerable number in any species can be labeled by the autoradiographic and immunocytochemical methods for GABA, the rest of the Purkinje cells remain unlabeled (8–11). In recent studies with *in vivo* injections of antibodies against GAD combined with anterograde and retrograde transport as a means for tracing chemically specific pathways (8), the significance of this patchy labeling of Purkinje cells was questioned. Does it mean that not all Purkinje cells contain GABA and GAD immunoreactivity, or that the GABA content in some Purkinje cells may be cyclic, so that GAD levels and therefore GAD immunoreactivity may fluctuate in time? If not all Purkinje cells contain GABA, then what are the other possible Purkinje cell neurotransmitters? A search among other neuroactive substances revealed that although monoamines and amino acids may characterize some fiber systems and may be candidates for transmitters of some cell types in the cerebellum, none of them applied to the Purkinje cells.

Recent studies indicate that motilin, a 22-amino acid polypeptide isolated from porcine gut 10 years ago (12), is present in the cerebellum (13). Motilin stimulates enteric smooth muscle (12, 14), has endocrine effects when administered systemically (15, 16), and has an excitatory effect on neurons of the cerebral cortex and spinal cord (17). Motilin-like immunoreactivity (motilin-i) is detectable in the brain of a number of species, particularly in the hypothalamus and pituitary, and less consistently in the cerebellum (18–21). The present investigation, in which double labeling techniques and polyclonal antibodies directed against the enzyme GAD and synthetic porcine motilin are used, demonstrates that these two substances can exist in Purkinje cells and even together in the same cells.

MATERIALS AND METHODS

The cerebellar cortex and deep cerebellar nuclei from adult mice, rats, and a monkey (*Macaca fascicularis*) were used in these experiments. The brains from these animals were perfused with 4% (wt/vol) formaldehyde in 0.12 M sodium phosphate buffer (pH 7.4) or with Bouin's picric acid/glacial acetic acid fixative, or they were immersed in the latter. The tissues were fixed overnight and prepared as follows: 50-μm-thick vibratome sections of formaldehyde-fixed tissue embedded in 7% agar, 6-μm-thick serial paraffin sections of Bouin's solution-

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Abbreviations: GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; GAD-i; GAD immunoreactivity; motilin-i, motilin-like immunoreactivity.

fixed material, 10-μm-thick cryostat sections of formaldehyde-fixed material. In several animals, colchicine, a drug that inhibits fast axonal transport (22), was given as a single intracisternal injection (30 μg/100 g of body weight) 24 hr prior to the perfusion.

GAD Antibody. GAD was isolated and purified from mouse brain, and the purity of the enzyme was established from the results of polyacrylamide gel electrophoresis, high-speed sedimentation equilibrium, and sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Antiserum to purified GAD was produced in rabbits by biweekly injections of 50 μg of the decarboxylase in complete Freund's adjuvant into the infrascapular muscles. The rabbits were bled 1 week after the fourth injection and afterwards. The GAD antiserum was characterized by enzyme inhibition, immunodiffusion, microcomplement fixation, and immunoelectrophoresis (23). The antisera used in these studies have been tested extensively in the cerebellar system (4–6, 6, 8).

Motilin Antibody. Synthetic porcine motilin (Peninsula Laboratories, San Carlos, CA) was conjugated to bovine thyroglobulin by the glutaraldehyde method (24), and the antiserum raised in rabbits by G.N. was characterized by radioimmunoassay (25) and found to be directed towards the mid-portion and COOH-terminal regions of the peptide. Preliminary immunostaining studies revealed positive labeling with this antibody in hypothalamus, pituitary, and cerebellum in rat, mouse, and man (13). Preincubation of the antiserum at a dilution of 1:1000 with 1 μg of synthetic porcine motilin abolished staining in hypothalamus and pituitary in rat. Preincubation of the antiserum at 1:1000 dilution with 100 μg of motilin resulted in reduced cerebellar staining. Reactivity was abolished in all processes, but 50% of reactive Purkinje cell somata remained. Preabsorption of this antiserum with rat cerebellar extract blocked staining in hypothalamus and pituitary as well as in cerebellum. The antigenic material in the cerebellum is distinct from but closely related to synthetic porcine motilin (13). It is possible that an antibody in the antiserum reacts with a particular steric conformation of the motilin molecule or even a large pre-motilin molecular form present in the sites of synthesis within Purkinje cell somata.

Histological Procedures. The process of double staining with the two antibodies, one against motilin and the other against GAD in sequential order, was applied to single sections so that each section was stained twice. The paraffin was removed from paraffin sections prior to hydration. All sections were rinsed in Tris·HCl buffer (0.01 M, pH 7.6) and treated with 0.5% H_2O_2 in Tris buffer for 30 min to abolish endogenous peroxidase activity before incubation with antibody. The most successful sequence was obtained with the initial use of motilin antibody followed by the GAD antibody. Motilin antiserum (dilution 1:400 to 1:600) in Tris buffer was used for primary incubation (overnight at 4°C or 2 hr at 37°C) in a humid atmosphere. The sections were then stained by the indirect peroxidase-antiperoxidase method (26) followed by diaminobenzidine visualization. Subsequently, the same sections were reincubated in the GAD antiserum (1:50 dilution, overnight at 4°C or 2 hr at 37°C), and stained by the indirect immunofluorescence method of Coons (27). The fluorophore used was either fluorescein isothiocyanate or rhodamine conjugated to goat anti-rabbit serum (1:50 dilution or 1:250 dilution). Glycerin was put on the sections, then coverslips; the sections were examined with fluorescence optics and with Nomarski interference optics. Another double-staining combination employed the immunoperoxidase method with different chromagens for distinguishing motilin-i from GAD immunoreactivity (GAD-i) by separate colors. In these sequences, motilin was revealed first by reaction with diaminobenzidine for a brown reaction product, and the same sections were then treated with GAD antibody bound to the same bridging secondary reagents but substituting 4-chloro-1-naphthol (28) to obtain a blue reaction product. Single representative sections of cerebellum in rat, mouse, and monkey, as well as sets of serial sections through rat and mouse brains each spaced 60 μm apart, were stained for mapping. For the present purposes, Purkinje cells staining with the anti-GAD serum were

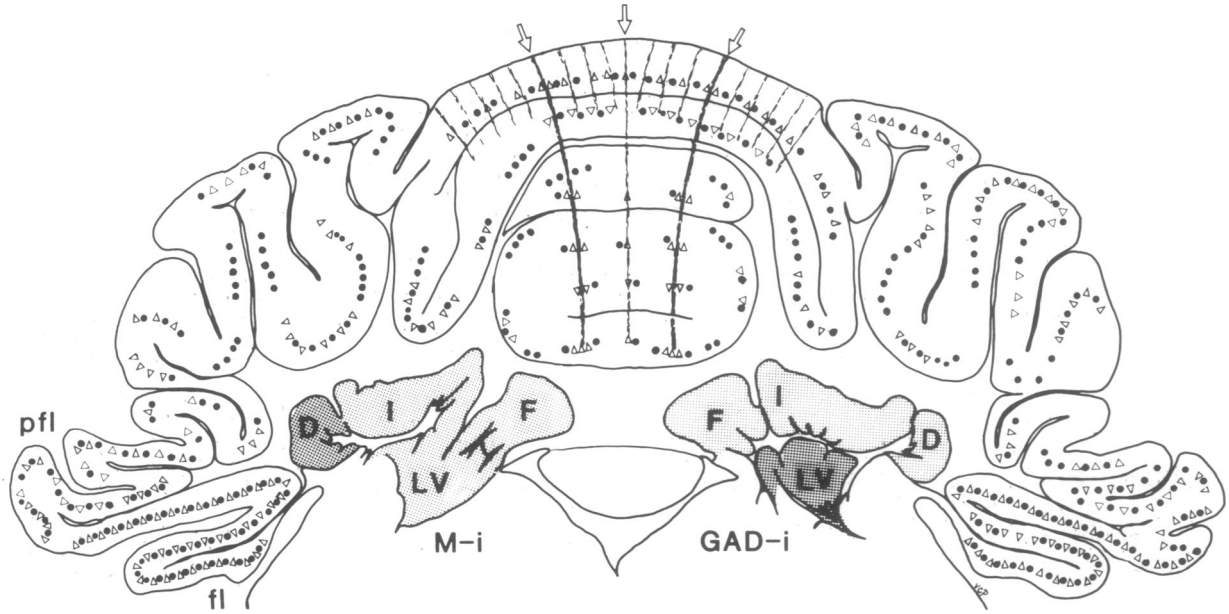

FIG. 1. Schematic drawing of the distribution of motilin-i (M-i) Purkinje cells (△) and GAD-i Purkinje cells (●) in a coronal section of the cerebellum. Motilin-i cells and GAD-i cells are both more concentrated in the flocculus (fl) and dorsal and ventral paraflocculus (pfl) than elsewhere. Both cell types occur in the vermis and participate in the formation of the sagittal microzones (arrows). Motilin-i terminal axon projections in the deep cerebellar nuclei—dentate (D), interpositus (I), fastigial (F), and lateral vestibular nucleus (LV)—are represented on the left and a comparable representation for GAD-i terminal axon projections is shown on the right. Motilin-i is heaviest in the dentate and GAD-i is heaviest in the lateral vestibular nucleus.

considered to be GABA-containing neurons, and those with motilin-i were considered to contain motilin or motilin precursor.

OBSERVATIONS AND DISCUSSION

M-i was observed in Purkinje cell somata, dendrites, and axonal plexuses in the cerebellar cortices of all three species examined—rat, mouse, and monkey—and in the terminal projections of Purkinje cells in the deep cerebellar nuclei and vestibular nuclei. The motilin Purkinje cells were most numerous in the flocculus and paraflocculus, where 60–70% of all Purkinje cells were immunoreactive. The lateral hemispheres had considerable numbers of these neurons arranged in large groups, punctuated by 1–12 or more Purkinje cells that were not motilin-immunoreactive. The paravermis and vermis of the cerebellum consistently contained fewer Purkinje cells with distinct motilin-i than did the lateral cerebellum. The motilin-Purkinje cells in the vermis were scattered singly or contributed to the formation of three narrow sagittal microbands in lobules I–IV. These three bands were precisely oriented with respect to the midline of the vermis (see Fig. 1)—a midsagittal band, two to four Purkinje cells wide in each folium and composed of the somata and dendritic trees lying precisely in the midline of the cerebellum, and two parasagittal bands, each approximately 100 μm from either side of midline and made up of the somata and dendrites of six to eight Purkinje cells both clustered and separate from one another. In lobules V and VI of the vermis, the three microzones were supplemented by a greater number (10–16) of narrower microzones each 50 μm wide, made up of one to three Purkinje cells. Regardless of their distribution, Purkinje cells with motilin-i were large cells, 20–35 μm in diameter, round or almost round, containing prominent nuclei that occupied much of the somatic volume, thus resulting in a high nucleocytoplasmic ratio (Figs. 2 and 3). The emergence of the primary dendrite was also prominent. Some Purkinje cells were intensely reactive, whereas others were less. Intracisternally administered colchicine did not increase the numbers of stained Purkinje cells but did increase the intensity of staining within somata and processes. Under closer examination, the motilin-i within somata occurred not only in the cytoplasmic matrix but also in the large Nissl bodies that these cells possess and in the protrusions of Nissl substance into the pale unstained nucleus (10). Varicose axons with motilin-i bordering the somata of Purkinje cells were best visualized on these somata without either motilin-i or GAD-i. These axons are likely to be the re-

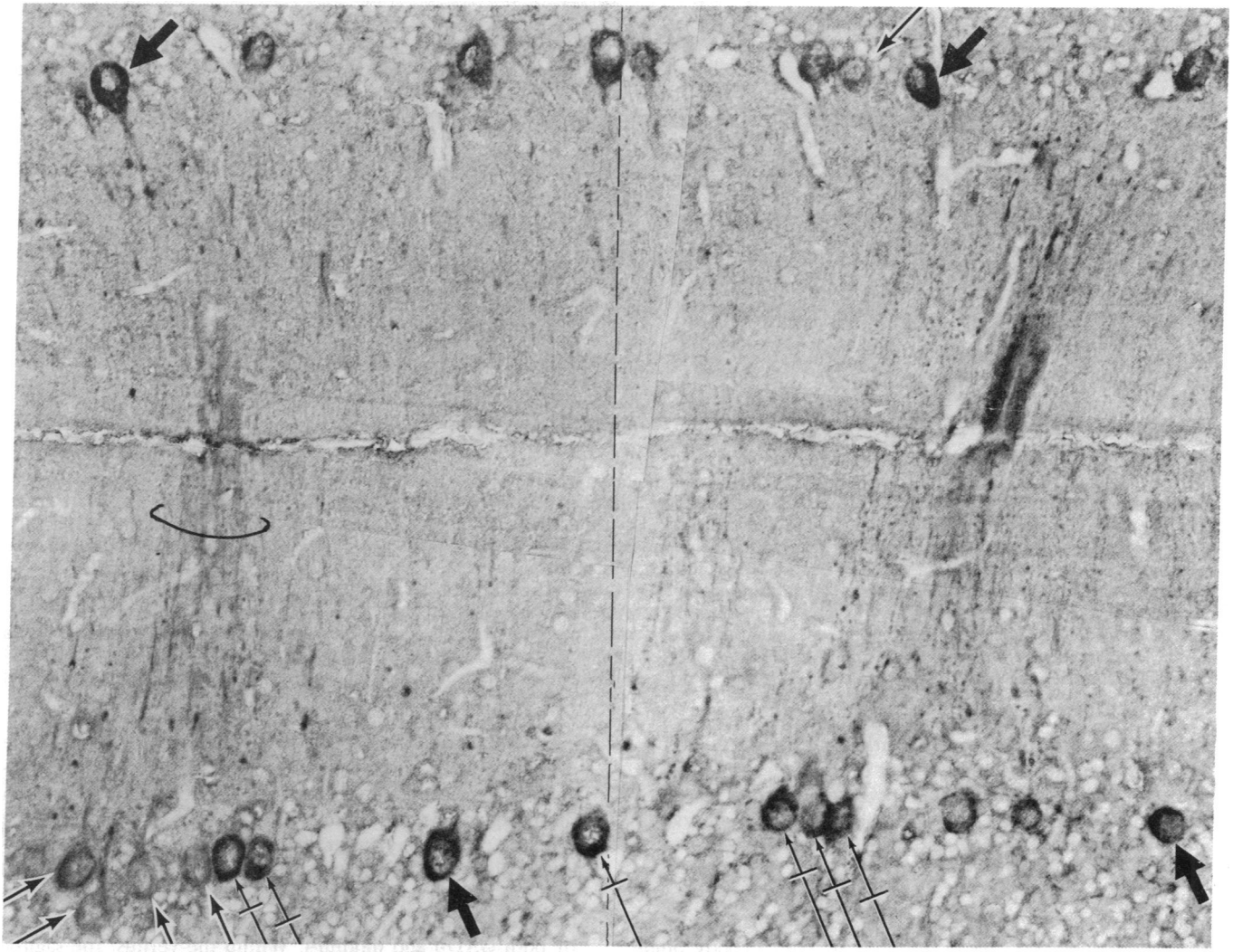

FIG. 2. Montage of photomicrographs showing the sagittal microzones in the midline (broken line) and on either side in the vermis of the mouse cerebellum. Motilin-i in Purkinje cells detected by immunoperoxidase techniques with diaminobenzidine (arrows) appears darker than GAD-i detected by chloronaphthol (crossed arrows). A number of cells have reaction products to both motilin-i and GAD-i and therefore appear black or darkest in this figure (large arrows). The microzones are formed by foci of motilin-i and GAD-i Purkinje cells together with their dendritic trees (bracket). (×100.)

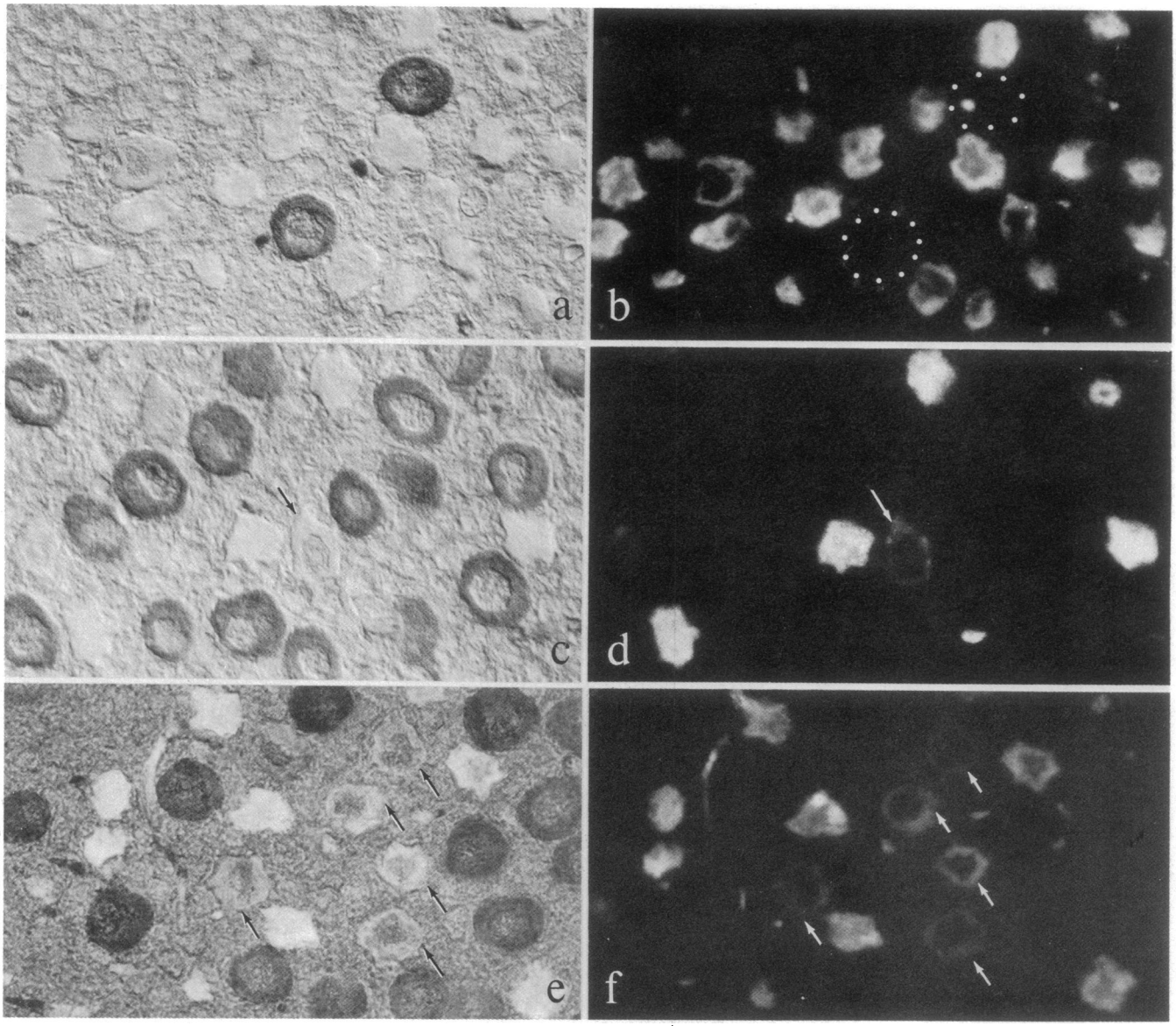

FIG. 3. (*a* and *b*) Purkinje cell field rich in GAD-i cells. A pair of photomicrographs showing the same field of Purkinje cells in which 2 of the largest neurons are labeled with motilin-i (*a*) and 21 of the remaining Purkinje cells are labeled with GAD-i (*b*). Note the variable intensity of GAD-i in these cells. The outlines of the two motilin-i cells not visualized by fluorescence are indicated by dots. [Mouse cerebellum lobule V, vermis, immunoperoxidase, Nomarski interference optics (*a*) immunofluorescence (*b*); both ×200.] (*c* and *d*) Purkinje cell field rich in motilin-i cells. A pair of photomicrographs showing the same field of Purkinje cells in which 17 large neurons are labeled with motilin-i (*c*) and 5 of the remaining Purkinje cells are labeled with GAD-i (*d*). Note again the differing intensity of GAD-i in one of these cells (arrow). [Mouse cerebellum, lobule V, vermis, immunoperoxidase, Nomarski interference optics (*c*); immunofluorescence (*d*); both ×200.] (*e* and *f*) Photomicrograph made by combining immunofluorescence and Nomarski interference optics on the same negative to show a Purkinje cell field with motilin-i cells (*e*, dark), GAD-i cells (*e*, light), and some neurons with both motilin-i and GAD-i (*e*, grey, arrows). The same field appears in *f* with fluorescence optics to show only GAD-i cells. Note that the neurons with lower levels of fluorescence (arrows) are those that contain both motilin-i and GAD-i (arrows). (Mouse cerebellum; ×200.)

current collaterals from axons of Purkinje cells with motilin-i, and other branches are also present in the granular layer. No other cell types of the cerebellum were observed to have motilin-i, the ony exception being a single large Golgi cell (rat) in which the dendritic and axonal plexuses were stained.

The Purkinje cells displaying GAD-i had a distinctive morphology (Figs. 2 and 3). These cells were generally slightly smaller than those with motilin-i, 15–25 μm in diameter, with nuclei that occupied a smaller proportion of the cell body. Some cells were round; the remainder were elliptical and even slightly irregular, each with a prominent primary dendrite. GAD-i Purkinje cells were found in considerable numbers throughout the cerebellum, filling in some of the spaces that were left vacant in the ranks by the motilin-i Purkinje cells. In the flocculus, paraflocculus, and cerebellar hemispheres, GAD-i Purkinje cells interdigitated with motilin-i Purkinje cells and both together accounted for many but not all of the Purkinje cells in these regions. In the vermal portions of the cerebellum, the Purkinje cell ranks of an entire folium contained either GAD-i or motilin-i, but were conspicuously punctuated by individual Purkinje cells or small foci belonging to the other neuromediator. Again many but not all Purkinje cells were accounted for by both GAD-i and motilin-i. Within the vermis, the sagittal microzones composed of motilin-i Purkinje cells were not obscured by the presence of associated GAD-i Purkinje cells. These studies confirm and extend the initial observations (13) that motilin antiserum demonstrates sagittal microzonation of a chemically defined neuronal population intrinsic to the cortex.

In previous descriptions, microzones were defined by the terminal projection fields of fibers projecting to the cerebellum, such as those from the inferior olivary nuclei, without definition of the chemical transmitter involved (29).

GAD-i was heavily concentrated in the Nissl substance as well as being present throughout the cell. As was described (8), the somatic staining for GAD ranged from intense to light and, although treatment with colchicine enhanced these levels, the relative differences in GAD content among individual neurons was preserved. GAD-i, unlike motilin-i, was not confined to Purkinje cells and their processes; many other cerebellar interneurons (basket, stellate, and Golgi cells) were labeled, as well as terminals and interneurons in the deep cerebellar and vestibular nuclei. The somata of Purkinje cells were surrounded by GAD-i axons that belonged to recurrent collaterals of other Purkinje cells or to basket axons.

Motilin-i and GAD-i were observed together in a small number of Purkinje cells scattered throughout the lateral and vermal cerebellum. In the double-stained preparations, in which a single cell had the labels for both motilin and for GAD, brown and blue, respectively, these cells were large, about 20–35 μm, round, with large nuclei, motilin-i generally in the somata and processes, and GAD-i heaviest in the Nissl substance. These cells probably accounted for about 10% of the total motilin-i and GAD-i populations (Fig. 2, Fig. 3 *e* and *f*). Together, GAD-i and motilin-i cells accounted for not more than 70% of the Purkinje cells in the lateral cerebellum and possibly 50–60% of the Purkinje cells in the vermal regions, thus suggesting that the remaining Purkinje cells in the cerebellum may contain neuroactive substances other than the two being investigated here.

The evidence presented indicates that cerebellar Purkinje cells are not a homogeneous population of neurons with a single inhibitory transmitter, GABA. Instead, these neurons are heterogeneous, both in morphology and in transmitter content—some are GABA neurons, others have motilin or pre-motilin and a small minority of the neurons appear to have both substances. A remaining population ranging between 30 and 40% of Purkinje cells has neither GABA nor motilin and is likely to have other neurotransmitter candidates. These results confirm earlier indications in the literature of the possibility of neurotransmitters in Purkinje cells other than the conventionally accepted one, GABA (8, 11). The observations indicate that individual Purkinje cells of the GAD variety may have greater or lesser amounts of this antigen and similarly those with motilin-i may have greater or lesser amounts of that antigen. Those with both substances may have variable amounts of each, some with more GAD, others more motilin/pre-motilin. As the examples of multiple neuroactive substances occuring together in single neurons increase, the present ideas on coexistence of transmitters will have to be expanded to encompass the two critical dimensions of time and function of the individual cell (30). Purkinje neurons that have motilin/pre-motilin and GABA may have fluctuating levels of one or both of these substances, according to some undetermined rhythm in the demands for one or the other mediating substance during cerebellar activity. The major factors that regulate such cyclicity and the manufacture, transport, and release of these substances, whether independently or concomitantly, need to be defined by further experimentation.

The recognition of motilin in Purkinje cells calls for an experimental definition of the role of this neuroactive substance in cerebellar circuitry and a reevaluation of the roles of Purkinje cells and of GABA in cerebellar function. Existing studies on the effects of motilin indicate a possible excitatory role in the cerebral cortex and spinal cord (17) quite different from the inhibition proposed for GABA in the traditional Purkinje cell cerebellar circuits. The fact that there is a significant motilin representation in the flocculus, paraflocculus and microsagittal zones in the vermis, and in the major innervation of appropriate deep cerebellar and vestibular nuclei, suggests that motilin may be a Purkinje cell mediator in the vestibular and eye movement-coordinating paths of the cerebellum. However, the distribution of GABA in the same areas indicates that the chemical preference may be at least bimodal.

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