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The unipolar brush cells of the rat cerebellar cortex and cochlear nucleus are calretinin-positive: a study by light and electron microscopic immunocytochemistry

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Abstract. Cell class-specific markers are powerful tools for the study of individual neuronal populations. The peculiar unipolar brush cells of the mammalian cerebellar cortex have only recently been definitively identified by means of the Golgi method, and we have explored markers of cerebellar neurons with the purpose of facilitating the analysis of this new cell population and, especially, its distribution and ultrastructural features. By light microscopic immunocytochemistry, we demonstrate that, in the rat, the unipolar brush cells are the cortical neurons that are most densely immunostained with antiserum to calretinin, a recently discovered calcium-binding protein. The unipolar brush cells are highly concentrated in the flocculo-nodular lobe, the ventral uvula and the ventral paraflocculus, occur at relatively high density in the lingula, at moderate-to-low density in other folia of the vermis and in the narrow intermediate cortex, and at low to very low density, with the exception of a few hot spots, in the lateral regions of the cerebellar hemispheres and in the dorsal paraflocculus. Unipolar brush cells are also found in the cochlear nucleus. In addition to the unipolar brush cells, calretinin antibody distinctly stains certain mossy fibers, and weakly to moderately stains other cerebellar elements, such as granule neurons and climbing fibers. In the lobules containing high densities of unipolar brush cells, the granule cell bodies and the parallel fibers are much less immunoreactive, and there are many more densely immunostained mossy fibers than in the lobules, where these cells are rare, which suggests some relationships between these elements. In the cerebellar nuclei, small neurons are densely immunostained, while large neurons are immunonegative.
The unipolar brush cells reside nearly exclusively in the granular layer. They are small neurons, intermediate in size between granule cells and Golgi cells, and their features are remarkably similar across all lobules. They usually have a single, relatively thick dendrite of varying length that terminates in a brush-like tip consisting of several short branchlets. Utilizing a pre-embedding protocol, we have identified unipolar brush cells with the electron microscope. The cytoplasm of these cells is partially obscured by the electron dense product of calretinin immunoreaction in all regions of the soma and processes. The cells are often covered with non-synaptic appendages and contain a peculiar cytoplasmic inclusion consisting of ringlet subunits. Other characteristic components are numerous neurofilaments, mitochondria and large, dense-core vesicles. Individual brushes enter one or two glomeruli, where the dendritic branchlets establish an unusually extensive synapse with mossy fiber rosettes. In addition to their contact with the mossy rosettes, the branchlets are postsynaptic to boutons presumably belonging to the axonal plexus of Golgi cells and are also presynaptic to small dendrites, displaying small, clear synaptic vesicles at the site of contact. The distinct calretinin-like immunoreactivity of the unipolar brush cells may be related to strong calcium influx at their extensive synapses with the mossy fiber rosettes.

Key words: Calcium binding proteins – Rat-302 cell – Pale cell – Golgi cell – Vestibulo-cerebellum

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Introduction

Progress in neurocytology has firmly established that neurons not only show an extraordinary degree of morphological diversity, but also substantially differ in their chemical make-up. Antisera to various cell surface and cytoplasmic molecules, coupled to sensitive immunocytochemical procedures, have provided new and powerful tools to classify neurons, to reveal the mode of distribution of distinct neuronal populations, and to analyze cell class-specific morphological features at both the light and the electron microscopic level. Among the molecules with cell class-specific expression, the calcium-binding proteins calbindin, parvalbumin, PEP-19 and calretinin have proved to be particularly advantageous in the study

of various regions of the central nervous system, including the cerebellum (reviewed by Rogers 1989b; Berrebi et al. 1991; Winsky and Jacobowitz 1991; Andressen et al. 1993).

Recently, we have become particularly interested in the unipolar brush cell (UBC), a neglected type of neuron in the cerebellar granular layer, newly uncovered with the Golgi method and other procedures (reviewed by Mugnaini and Floris 1994). These cells are particularly numerous in the vestibulo-cerebellum and the vermis, and may be part of neuronal microcircuits subserving special motor control mechanisms. In the older literature, UBCs may have been confused with Golgi type II neurons, which are local circuit neurons of the granular layer.

In this paper, we demonstrate that antibody to calretinin is a powerful marker for the rat UBCs, we map their distribution, and establish criteria for their identification in the electron microscope utilizing a pre-embedding protocol. In addition, we show that various grades of calretinin immunoreactivity are present in various cells and fibers of the rat cerebellum and evaluate the usefulness of calretinin antiserum as an immunocytochemical marker. Some of these data have been presented elsewhere in preliminary form (Floris et al. 1992).

Materials and methods

The materials for this study are derived from 15 adult male and female Sprague-Dawley rats, 250–400 g in body weight. The animals were housed in supervised animal facilities and handled according to approved guidelines.

Procedure for light microscopic immunocytochemistry

The rats were deeply anesthetized with 40 mg/kg sodium pentobarbital, i.p., and perfused through the ascending aorta (Friedrich and Mugnaini 1981), first with saline and then with zinc-formol in saline (Mugnaini and Dahl 1983), at room temperature. The cerebella were removed 2 h after perfusion and immersed in chilled saline with 30% sucrose until they sank, for cryoprotection. They were then sectioned at 30 μm on a freezing microtome in the sagittal, coronal, and horizontal planes. The free-floating sections were first immersed for 1 h in a blocking solution consisting of 5% dried non-fat milk and 0.5% Triton-X 100 in 0.5 M TRIS-HCl buffer, pH 7.6, and then immunoreacted for calretinin following the peroxidase-antiperoxidase (PAP) protocol, with the specific antiserum (diluted 1:1,000) previously characterized (Winsky et al. 1989; Dechesne et al. 1991), and utilizing DAB as the chromogen (reaction time 17–25 min). Some of the immunoreacted sections were postfixed for 3 min in 0.005% osmium tetroxide in water to enhance contrast. Sections were dry-mounted on glass slides and coverslipped with Permount. Control sections incubated with preimmune rabbit serum were completely free of immunoreaction product, as previously documented (Winsky and Jacobowitz 1991).

Immunoreacted serial sections of the cerebellum cut in the horizontal and the sagittal planes were outlined on paper at low magnification with a drawing tube attached to the light microscope. Individual, calretinin-positive cell bodies in the granular layer, intermediate in size (9–12 μ m in diameter) between granule and Golgi cells, were identified under a × 40 objective lens and their positions marked with dots on the drawings. All such immunostained cell bodies were marked, whether or not the cell nucleus or a brush-bearing dendrite was included in the section, on the assumption that they represented UBCs. Multipolar, calretinin-positive neurons were not included.

Procedure for immuno-electron microscopy

Five rats were perfused at room temperature, with an oxygenated Ringer solution, followed by a fixative containing 4% freshly depolymerized formaldehyde, 0.1% glutaraldehyde, and 0.12 M sodium phosphate buffer, pH 7.3, and then by a second fixative identical to the above, except for the omission of glutaraldehyde. The animals were kept refrigerated for 1 h after perfusion, and then the cerebella were removed and sliced on a Vibratome at 40–45 μ m. The slices were immunoreacted free-floating for calretinin, according to the PAP protocol indicated above, but with three alterations: the detergent was omitted, 0.12 M phosphate buffer was used as diluent and rinsing solution, and the DAB reaction was carried out for only 4–6 min. The Vibratome slices were rinsed thoroughly, postfixed in buffered 2% osmium tetroxide, rinsed in distilled water, postfixed in 1% aqueous uranyl acetate, rinsed again, dehydrated and embedded flat in an Epon/TAAB resin mixture between two acetate sheets. After polymerization for 2 days at 60°C, the slices were re-embedded in Epon blanks before ultramicrotomy. Ultrathin sections, collected on Formvar-coated single-hole grids, were lightly contrasted with lead citrate for 3 min and photographed on a Zeiss EM-10 electron microscope operated at 80 kV.

Results

Light microscopic immunocytochemistry

Under low-power lenses, it is immediately apparent that calretinin immunoreactivity is displayed to varying degrees by different cerebellar components and that the immunostaining shows distinct topographic variations within the same specimen (Fig. 1). Two classes of cerebellar cortical components appear most intensely immunostained (Fig. 2): mossy fibers and their glomerular endings, and peculiar small neurons of the granular layer identical to the unipolar brush cells (UBCs), previously demonstrated with various procedures, including the Golgi method (Mugnaini and Floris 1994). All other immunoreactive cortical elements including granule cells, parallel fibers, climbing fibers and some of the inhibitory interneurons, are usually stained much more weakly. Purkinje and stellate cells are always immunonegative. There is also substantial immunostaining of neurons and fibers in the cerebellar nuclei (Fig. 4), the folial and deep white matter, the cerebellar peduncles, and the adjacent cochlear and vestibular nuclei.

Cerebellar cortex

Figures 1 and 3 illustrate the varying degrees of calretinin immunoreactivity in parasagittal sections through the median cerebellar cortex. Immunoreactive UBCs and mossy fibers (Fig. 2) stand out among the less distinctly stained elements.

Calretinin-positive mossy fibers

Strongly immunoreactive mossy fibers (Figs. 2, 3 B), as sharply marked by the immunoreaction product as in Golgi-impregnated sections, are seen at high density in the flocculus, the ventral paraflocculus, the nodulus and

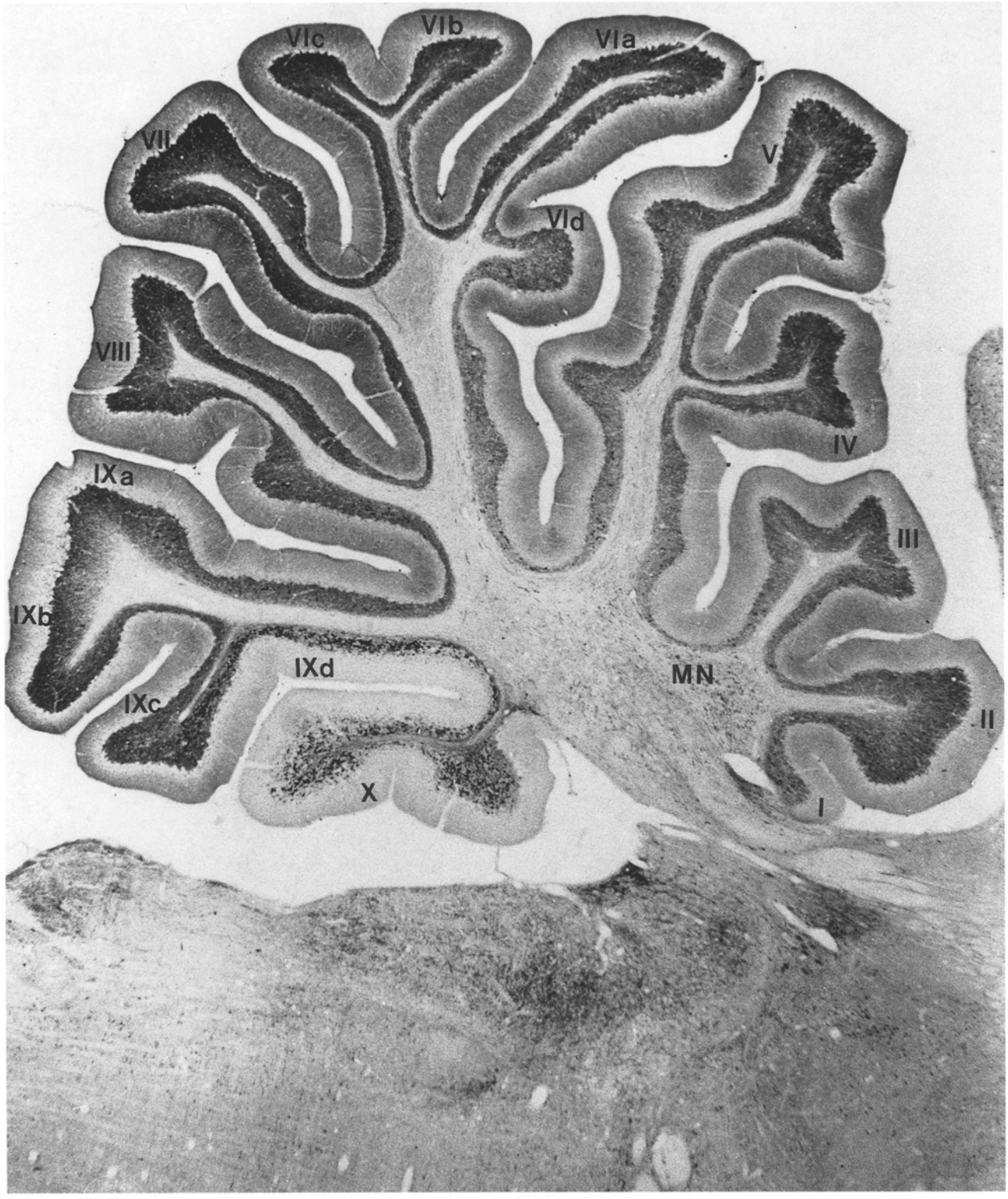


Fig. 1. Parasagittal section of the rat cerebellar vermis immunostained with antiserum to calretinin. Folia are labeled with roman numerals according to Larsell. The pattern of immunoreactivity in the nodulus (lobule *X*) and portion of the ventral uvula (lobule *IXd*) differs from that in the other vermal folia. While in most of the vermis weak immunoreactivity is present in granule cell bodies within the granular layer and in parallel fibers within the molecular layer, in the folia of the vestibulo-cerebellum the immunoreaction product is largely localized in special elements of the granular layer that, at higher magnification (see Fig. 2), are identified as unipolar brush cells (UBCs) and mossy fibers. *MN*, Medial cerebellar nucleus. × 28.5

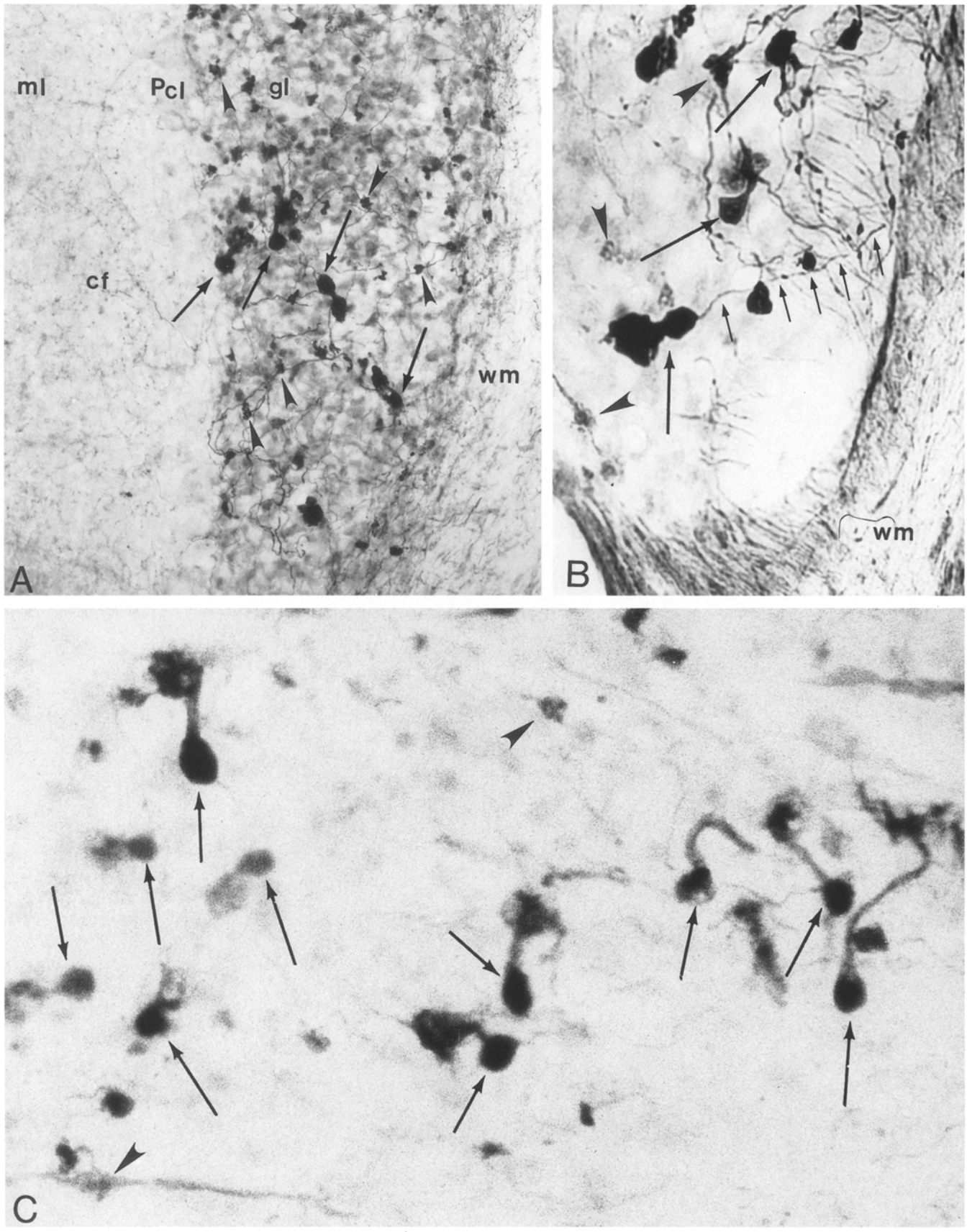


Fig. 2A–C. UBCs (*arrows*) and mossy fibers (*arrowheads*) are the main calretinin immunoreactive elements in the cerebellar cortex. *ml*, Molecular layer; *Pcl*, Purkinje cell layer; *gl*, granular layer; *wm*, white matter. **A** Ventral uvula. Moderately stained climbing fiber terminals, *cf*; numerous immunopositive thin fibers and scattered thick fibers in the white matter are unlabeled. × 350. **B** Nodulus. The axon (*thin arrows*) of a densely stained UBC in the nodulus can be followed until it enters the white matter. × 500. **C** Nodulus. UBCs emit a relatively large dendritic trunk that terminates with a brush-like spray of branchlets; the thin axon, which can be followed only for a short distance, is apparent in the cell at the *top left*. × 700

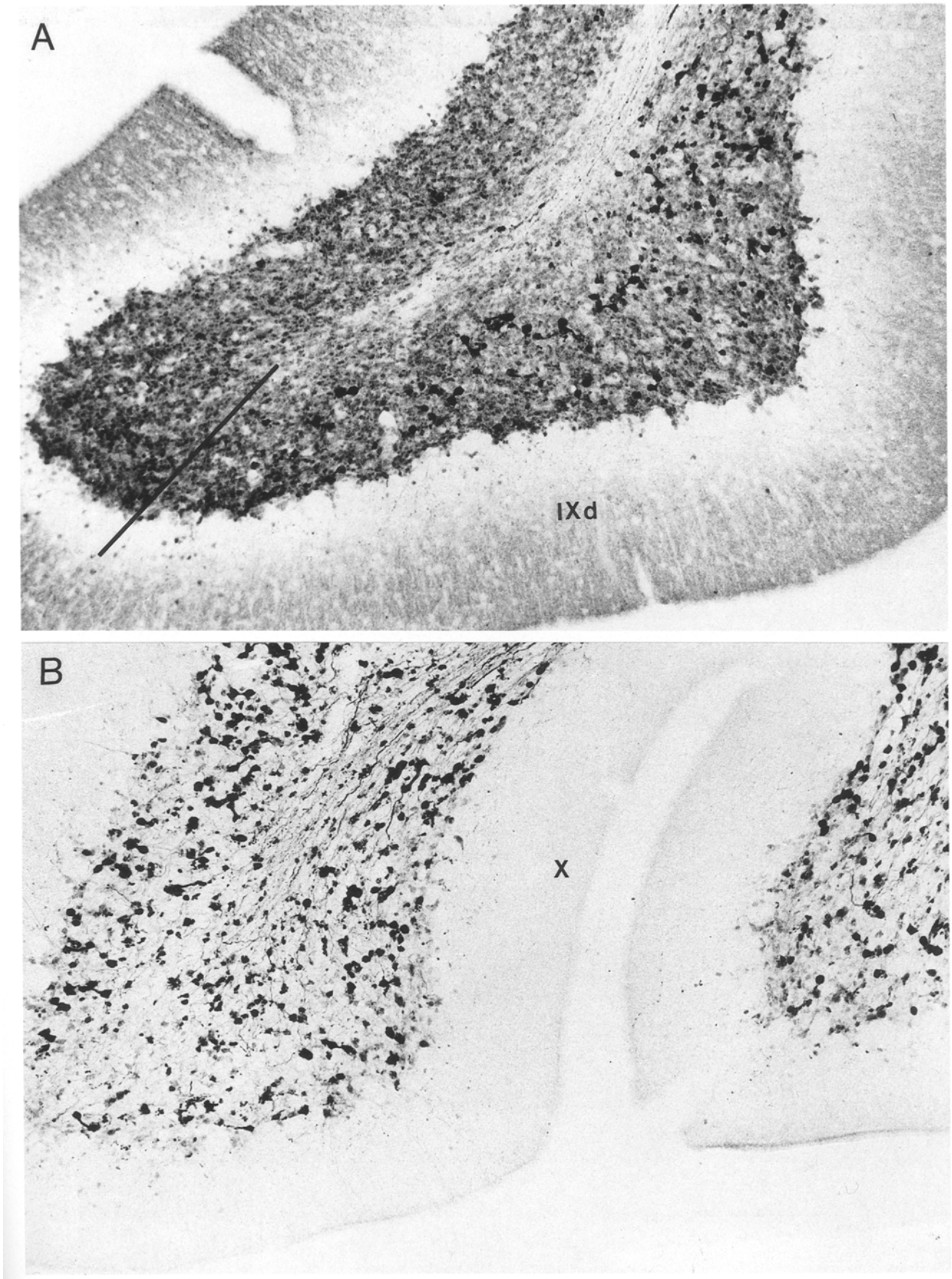
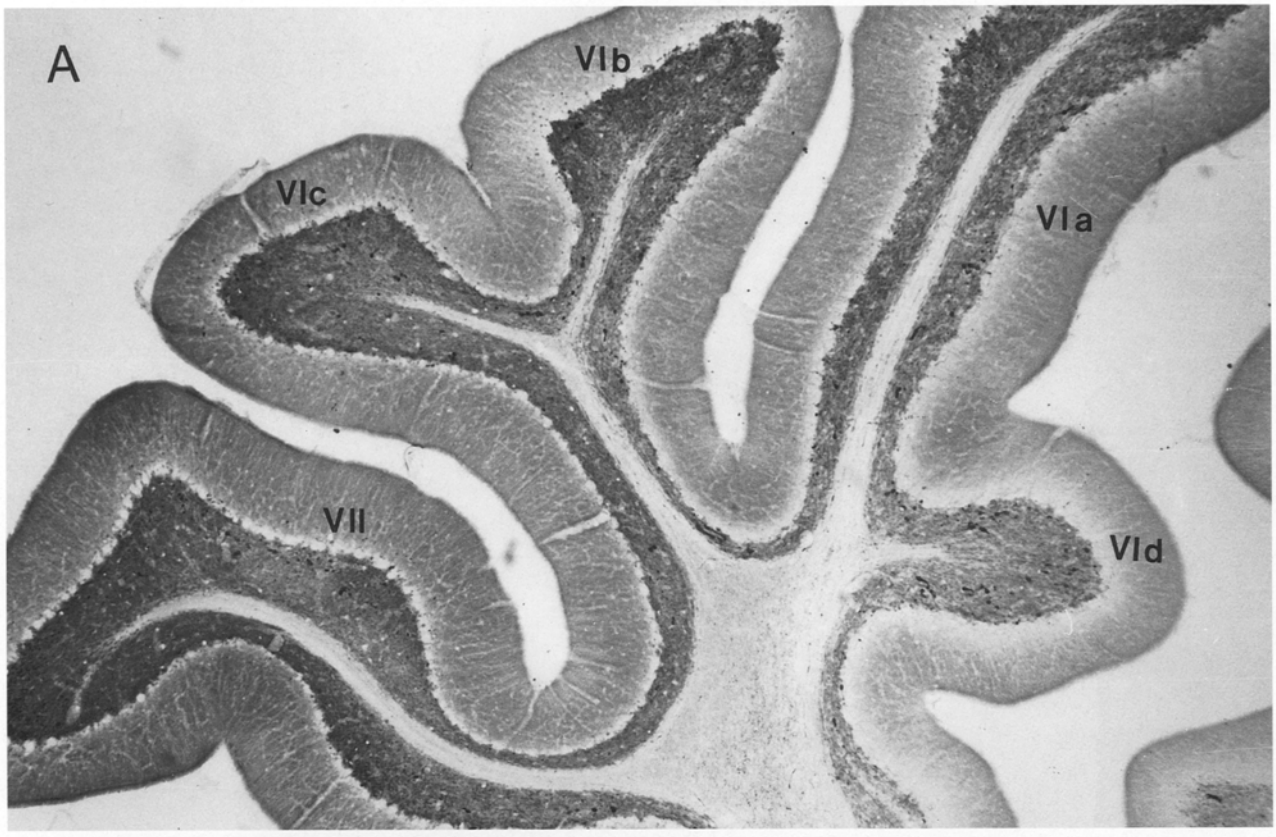


Fig. 3. Changing patterns of calretinin immunoreactivity (details from a sagittal section analogous to that illustrated in Fig. 1). **A** Ventral uvula, and **B**, nodulus. The line in **A** demarcates the UBC-rich zone (*right*) from the UBC-poor zone. × 230


A
VIb
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VII
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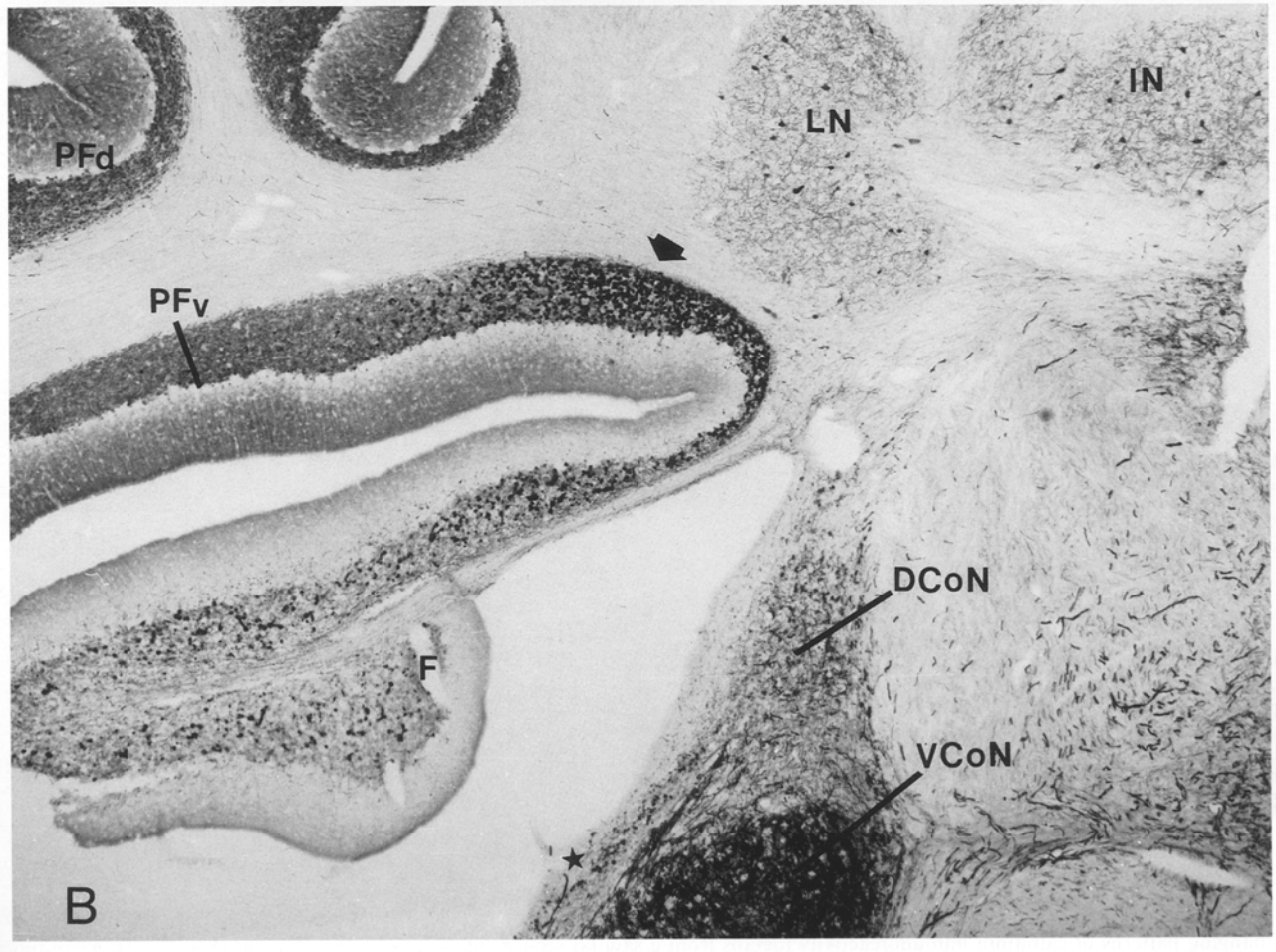
PFd
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the ventral uvula (IXd), and at lower density in lobules I (lingula) and II. All the other vermal lobules and the hemispheres contain only a few, if any, mossy fibers that are densely calretinin-positive. However, a low density of staining in the glomeruli is present in most of the folia, especially in sections incubated for 25 min in DAB/H2O2.

Distribution of the unipolar brush cells

In median parasagittal sections (Figs. 1, 3), the highest density of UBCs occurs in the nodulus (lobule X) and the ventral uvula (lobule IXd), followed in decreasing order by the lingula (lobule I), the parts of lobules IV and VI around the deep fissures (Fig. 1) and the folia of lobule VI (Fig. 4 A), and lobules VII and VIII. In coronal sections of the cerebellum, UBCs are also observed at high densities in the flocculus and the ventral paraflocculus (Fig. 4 B). At the transition between flocculus and ventral paraflocculus (thick arrow in Fig. 4B) there is a particularly high concentration of densely stained small cells, which we provisionally interpret as UBCs, pending electron microscopic verification. Analysis of serial sections of the cerebellum indicates that, in general, the concentration of UBCs drops drastically as one progresses from the vermis to the lateral cortex; it is moderate in the thin intermediate cortex and very low in the lateral cortex. This trend is clearly apparent from the cameral lucida drawings of serial, sagittal and horizontal sections shown in Figs. 5 and 6. There are, however, certain peculiarities to be noted. The concentration of presumed UBCs, which are represented by dots, is higher in the lateral portions of the anterior lobe, crus I, the copula, and the paramedian lobule, than in crus II and the dorsal paraflocculus. There are small hot spots of densely packed UBCs outside the flocculo-nodular lobe and the vermis; the most remarkable of these is situated bilaterally and symmetrically, in the lateralmost folia of crus I (H-VII) (Fig. 7). Also, the concentration of UBCs remains relatively high in the most lateral regions of the anterior lobe folia (Fig. 5). This situation is photographically illustrated in Fig. 8 A for lobule II. Because the cerebellar cortex is known to contain sagittally organized compartments, or zones (Voogd and Bigaré 1980), we examined coronal and horizontal sections to see whether the distribution of UBCs and calretinin-positive mossy fibers showed clear signs of zonation. In some sections, we observed UBC-rich and UBC-poor compartments comprised within the vermis and the intermediate cortex (Fig. 8 A), but the zones of apparent UBC abundance were usually evident only in lobules II and III. In other sections, zones of lesser calretinin immunoreactivity in one folium were not in register with those of a neighboring folium, or they were not repeated in successive sections. No sign of sagittal compartmentation is present in coronal and horizontal sections of the lingula and the nodulus (Fig. 8 B), which are regions of high density of immunostaining. We concluded, therefore, that zonation of UBCs is not prevalent and may occur only in restricted cortical regions. Furthermore, the impression of banding may sometimes be engendered by technical artifacts, such as adherence between sections in the wells during immunostaining.

An unsolved problem of cerebellar microanatomy is whether superficial and deep levels of the granular layer differ in composition. This has especially been discussed with respect to the distribution of mossy fiber afferents from different sources (Eccles et al. 1967; Ito 1984), but it could also apply to the UBCs. While in most folia calretinin-positive mossy fibers and UBCs occupied all levels of the granular layer, in some folia they were preferentially distributed beneath the Purkinje cell layer (Fig. 7) or near the white matter. In some of the folia, like H-VII, the superficial distribution of UBCs was similar in different rats (Fig. 7), but in other folia the special distribution of UBCs in one specimen was not exactly the same as that in another specimen. This is consistent with the assumption that there are minor individual variations, although these remain to be ascertained with exacting analyses. Notably, a few UBCs are present in the posterior medullary velum (not shown) and are irregularly scattered along the ependyma lining the roof of the fourth ventricle (Fig. 9 C). These neurons are present in all our specimens, albeit at varying concentrations, and presumably represent displaced, or "ectopic", cells.

Cytology of the UBCs. The UBCs reside in the granular layer and the underlying white matter. They are small neurons (Fig. 2) intermediate in size between granule cells and Golgi cells (Fig. 9). Their small, round or oval cell bodies, 9–12 μ m in diameter, in many instances appear to give rise to a single, short and stout dendrite, 2–3 μ m in diameter, that terminates with a brush-like spray of short branchlets. The total length of the dendrites ranges from 5 to 50 μ m, but in most cells it measures between 10 and 30 μ m. We occasionally encountered cells with two such dendrites or with a single dendritic stem dividing into two branches each giving rise to a brush-like tip. In one case we saw a cell with one brush originating from the cell body and one dendrite dividing once to form two

Fig. 4. Parts of the vermis, the flocculus, the paraflocculus and the cerebellar nuclei (from sagittal, **A**, and coronal, **B**, sections of the cerebellum immunostained with calretinin antiserum). Of the most densely stained elements, the larger ones represent UBCs and the smaller mossy fiber endings. The *gray tones* in the granular and molecular layers represent weakly immunostained granule cell bodies and parallel fibers. **A** The micrograph includes parts of five median cerebellar folia, four belonging to lobule VI and one to lobule VII, that contain scattered UBCs. **B** The micrograph includes parts of the flocculus (*F*), ventral paraflocculus (*PFv*), dorsal paraflocculus (*PFd*), lateral cerebellar nucleus (*LN*), interposite cerebellar nucleus (*IN*), and the dorsal (*DCoN*) and ventral (*VCoN*) cochlear nuclei. The superficial granule cell domain of the VCoN is indicated by a star. Note the extremely high density of densely stained small cells at the transition flocculus/paraflocculus (*thick arrowhead*). The cerebellar nuclei contain densely stained small neurons and their dendritic and axonal plexuses. In the cochlear nuclei, the densely stained elements are cochlear nerve fibers and different categories of neurons. At higher magnification, some of the positive cells in the DCoN and the superficial granule cell domain were identified as UBCs (see Fig. 10). × 77

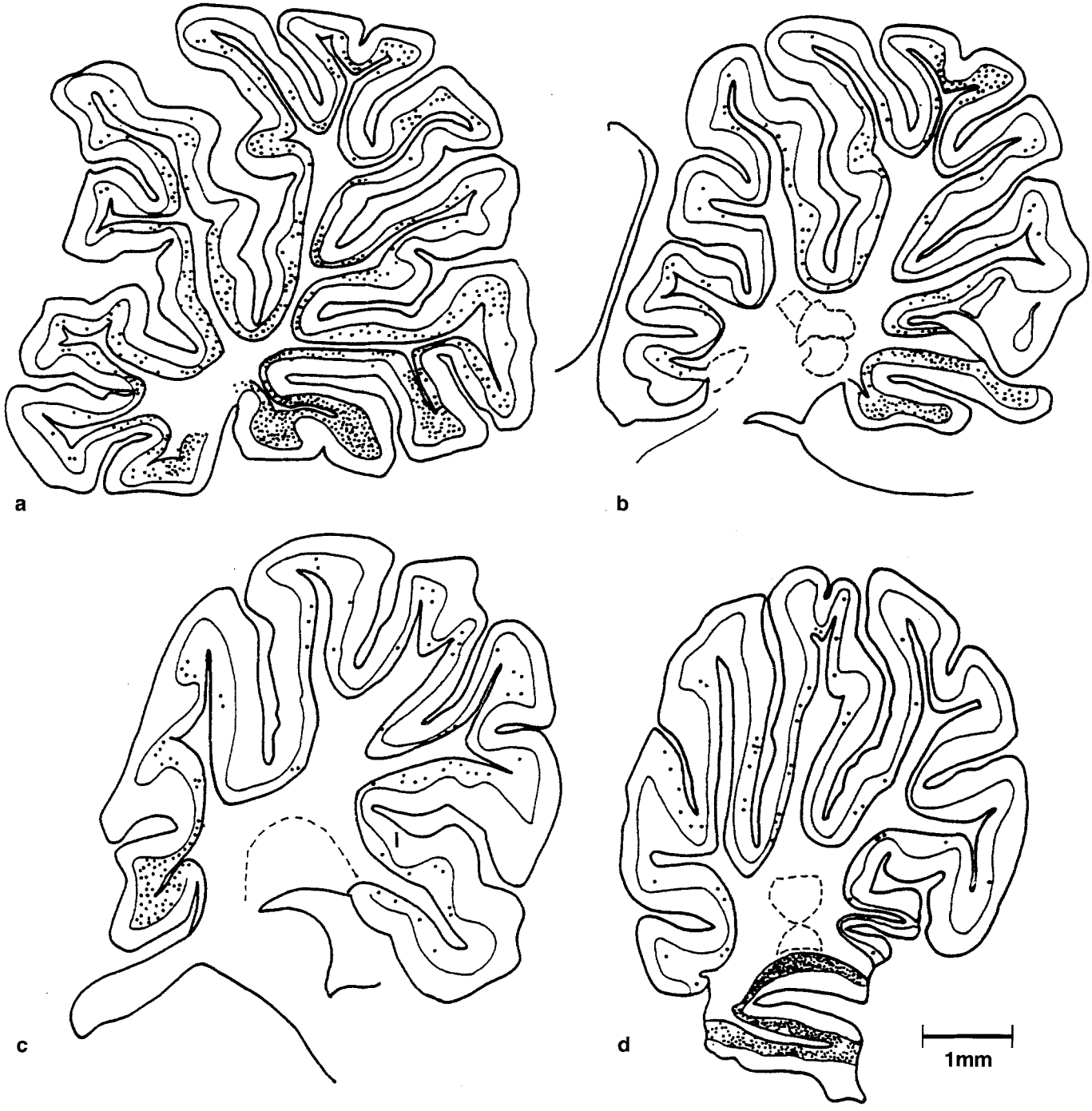

Fig. 5. Camera lucida drawings of serial parasagittal sections of the cerebellum showing the distribution of presumed UBCs (*dots*)

other brush tips. Brush-tips and calretinin-positive mossy rosettes may form compact nests, suggesting that they articulate synaptically. The UBC emits a single thin axon, which usually arises from the aspect of the perikaryon opposite the dendritic base (Fig. 2B, C) and is often difficult to trace (Fig. 2 C). In some of the cells situated nearest the white matter (Fig. 2 B) or within the white matter itself, the thin axons assume a course parallel to the afferent and efferent fibers within the white core of the folium and were occasionally followed for up to 250 μm.

Granule neurons. As shown in Figs. 1 and 3, the intensity of staining of the granule cells and the parallel fibers in the vermis shows moderate gradients: it increases lightly from lobule I to lobules VI and VII, where parallel fiber staining is maximal, drops gradually towards lobule IXa (the dorsal uvula), and then decreases sharply in lobule IXc, to become minimal at the bottom of IXd and in lobule X. In the hemispheres the intensity of staining matches that of the posterior cerebellar lobules VIII-IXa, while at the transition between the ventral paraflocculus and the flocculus (Fig. 4 B) it drops sharply, matching the

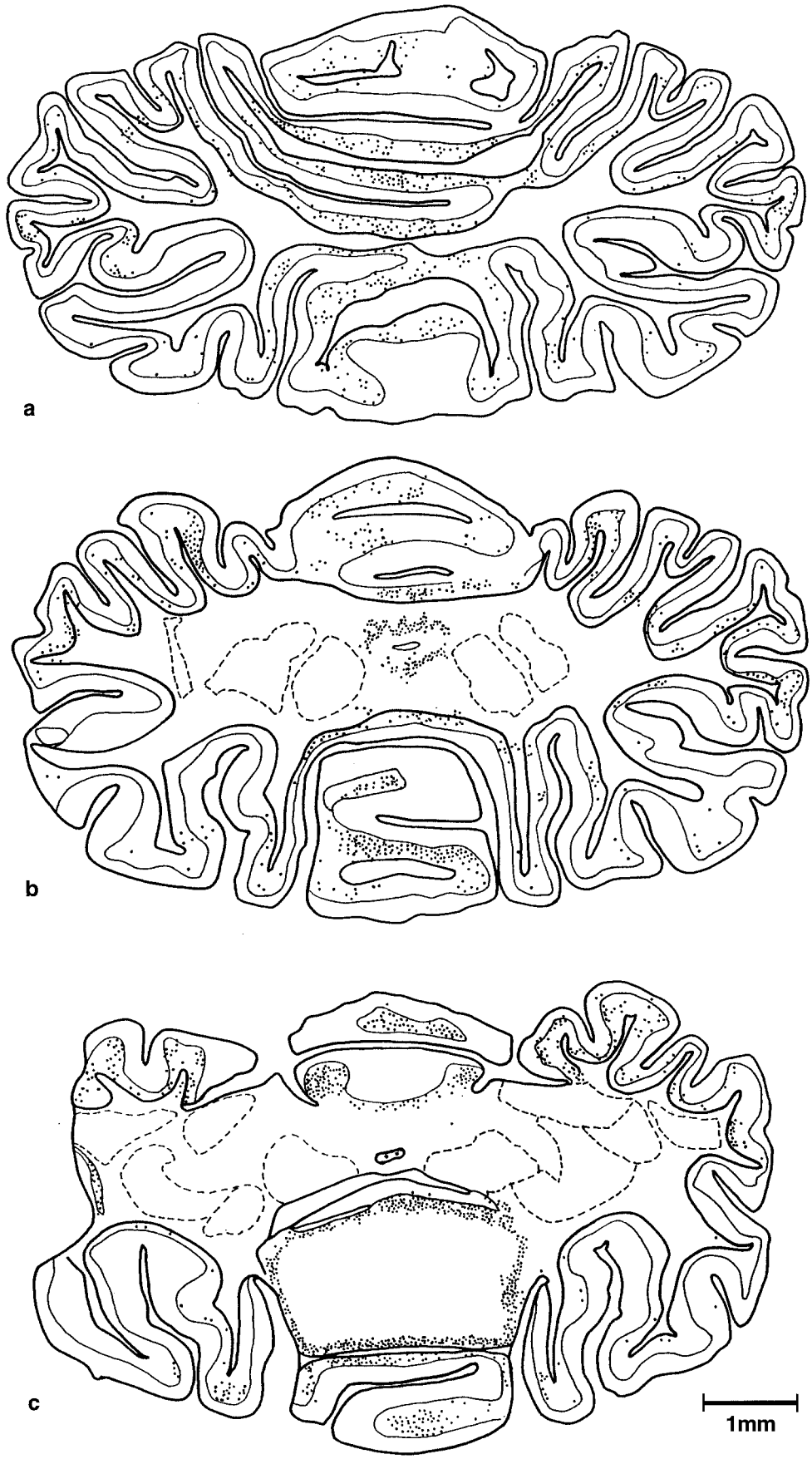

Fig. 6. Camera lucida drawings of serial horizontal sections of the cerebellum showing the distribution of presumed UBCs (*dots*)

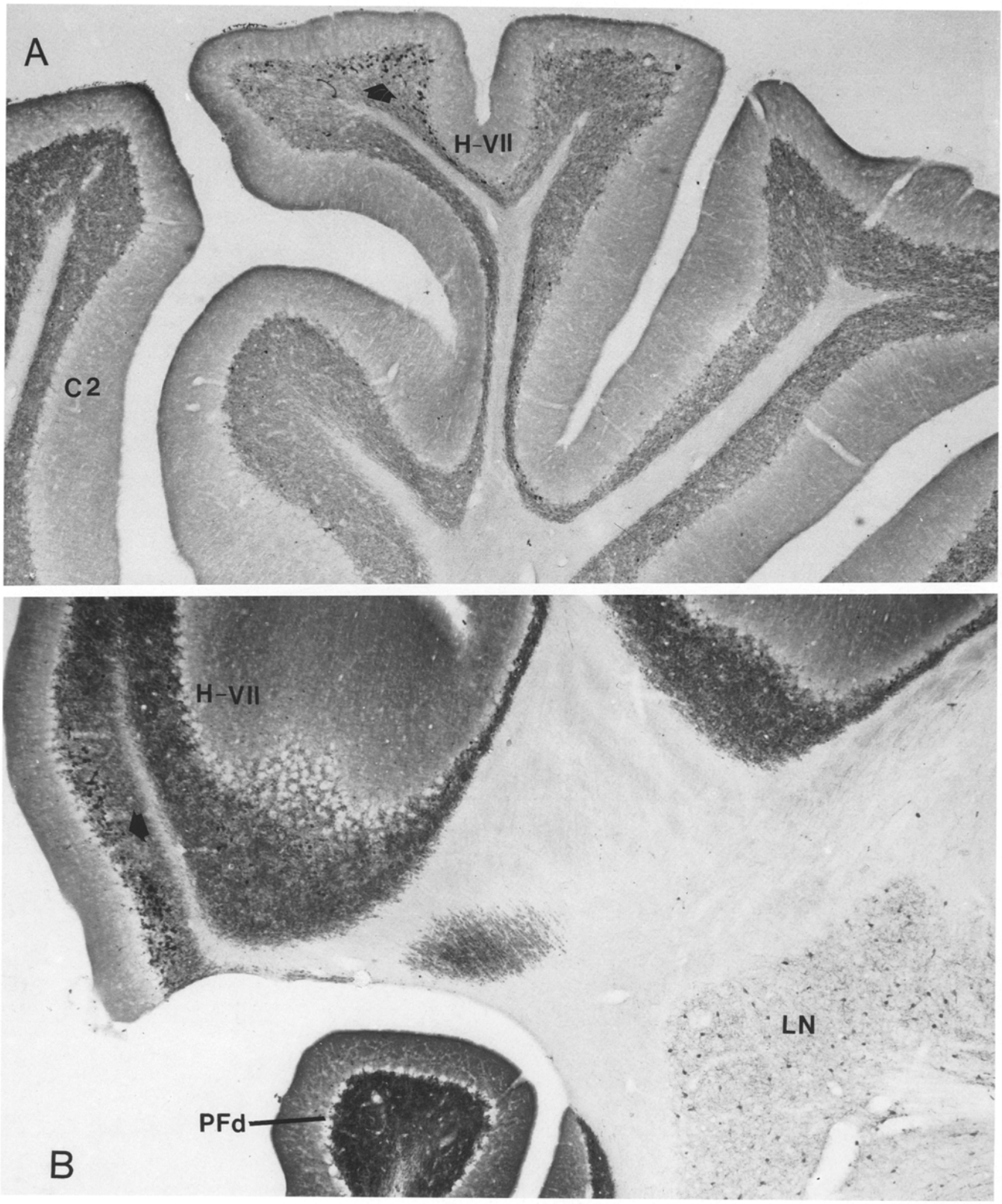

Fig. 7. UBC hot spots. A hot spot of UBCs is present in the superficial region of the granular layer of lobulus H-VII of crus I (*thick arrowhead*), identified in horizontal (**A**) and coronal (**B**) sections, while UBCs are rare in neighboring folia of crus 1 (unlabeled), crus 2 (*C2*), and in the dorsal paraflocculus (*PFd*). *LN*, Lateral cerebellar nucleus. × 340

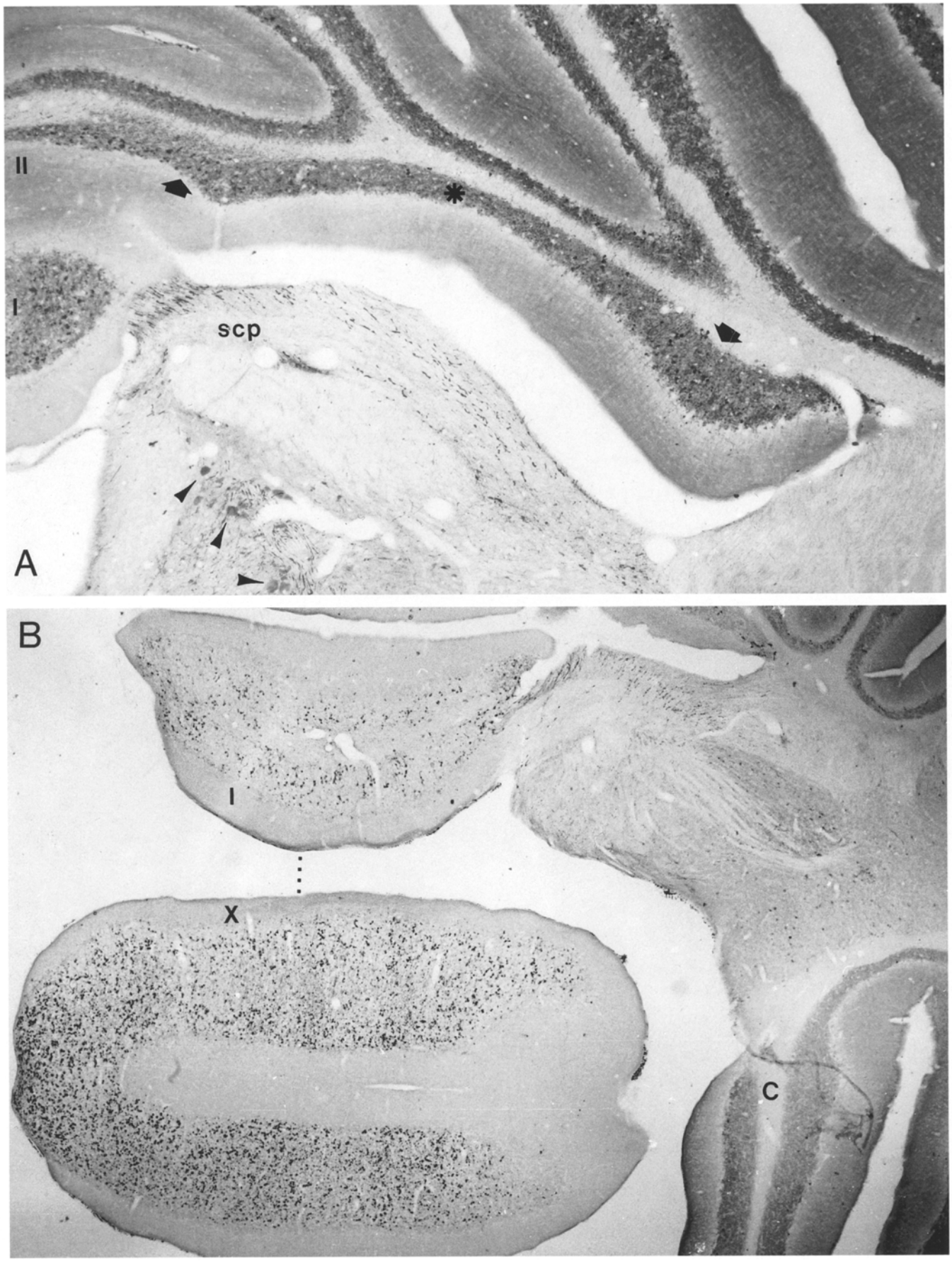

Fig. 8. Mode of distribution of UBCs in coronal (**A**) and horizontal (**B**) sections of lobules I, II, and X. **A** In this section, lobule II seems to include two zones of the granular layer with abundant UBCs (*thick arrows*), separated by a UBC-poor gap (*asterisk*) situated in correspondence with the intermediate cortex. The midline is at the left border of the picture. The superior cerebellar peduncle (*scp*) contains densely immunoreactive fibers. *Arrowheads* point to moderately stained cell bodies of the mesencephalic trigeminal nucleus. **B** No gaps are apparent in the distribution of UBCs in the nodulus (*X*) and the lingula (*I*). The midline is indicated by a *dotted line*. The copula (*C*) contains scattered UBCs. × 270

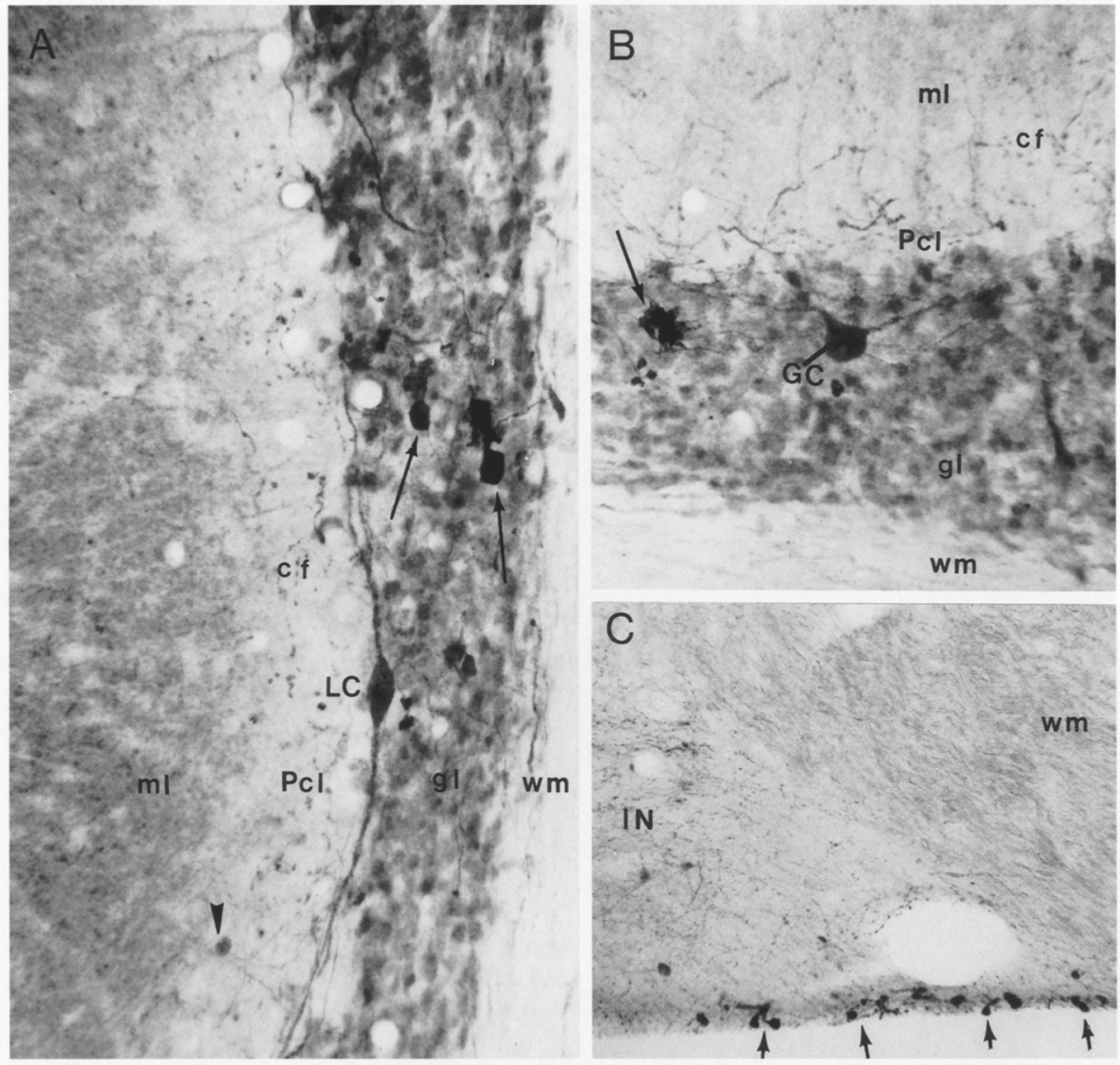

Fig. 9. Calretinin-immunoreactive elements in the cerebellar cortex (**A, B**) and along the roof of the fourth ventricle (**C**), after osmium intensification. *ml*, Molecular layer; *Pcl*, Purkinje cell layer; *gl*, granular layer; *wm*, white matter; *IN*, interposite nucleus. **A, B** Granule cells are weakly stained and UBCs (*arrows*) are densely stained. A fusiform Lugaro cell (*LC*) and a large Golgi cell (*GC*) are stained less densely than UBCs and mossy endings. A basket cell body (*arrowhead*) and parallel fiber varicosities (unlabeled *grey portion of ml*) are faintly stained. Scattered climbing fiber boutons (*cf*) are moderately stained. × 400. **C** Densely stained, ectopic UBCs (*arrows*) are aligned along the ependyma of the fourth ventricle. × 170

pattern observed in the nodulus. These patterns were present in all the rats.

Climbing fibers. Climbing fiber terminals are evident only when the immunoreaction is pushed to its limit; generally, they are recognized where they enter the molecular layer and begin to arborize, but they can not be individually followed to higher levels of the molecular layer because of insufficient staining and/or masking of the climbing fiber varicosities by parallel fiber boutons. Climbing fiber arbors are distinguished more easily in the vestibulo-cerebellum, where they are somewhat more densely stained and the parallel fiber staining is extremely weak (Fig. 9 A, B). This is in accord with the varying degree of immunostaining of the parent inferior olivary neurons (data not shown).

Other cortical neurons. Some multipolar cells, 12–20 m in cell body diameter (Fig. 9 B), occurring at all levels of the granular layer (presumed Golgi type II cells), and

fusiform cells, 15 μ m in cell body diameter (Fig. 9 A), situated just beneath the Purkinje cell layer (presumed Lugaro cells; Sahin and Hockfield 1990), usually appear weakly to moderately stained. Only occasionally did we encounter Golgi and Lugaro cells that appeared densely calretinin-positive with the standard protocol used in this investigation. In the sections where the immunoreaction product had been intensified with osmium (Fig. 9A), the basket cells appear faintly immunoreactive, especially in the lingula, nodulus and flocculus, where parallel fiber staining is weakest. Basket cells, however, are usually unstained in most of the folia in sections immunoreacted with the standard protocol.

Cerebellar nuclei. In all four cerebellar nuclei there are scattered, densely immunopositive neurons (Figs. 1, 4 B, 7 B, 8 B). These have small to medium cell bodies (12–14 μ m), with dendrites usually distributed in a stellate configuration. A diffuse axonal plexus (Fig. 4 B), presumably belonging to these neurons, is also distinctly stained, as are a number of large afferent fibers. The large projection neurons usually appear immunonegative or, at most, weakly stained.

White matter. Bundles of immunostained fibers of varying diameters are observed in the folial and deep *white matter* and in the cerebellar peduncles (Figs. 1, 3, 4 B). The largest fibers are undoubtedly primary vestibular afferents, because they can be followed from their strongly immunoreactive perikarya in the Scarpa's ganglion (not shown), to the eighth nerve root, the vestibular nuclei, the nodulus and the lingula. The other fibers may have different origins, because a variety of other precerebellar nuclei contained immunoreactive neurons (data not shown).

Cochlear nucleus. Cells identical to the cerebellar UBCs are also found in the cochlear nucleus (Fig. 10). In the ventral cochlear nucleus (VCoN) these cells occur in the superficial granular layer (Fig. 10 A), the subpeduncular corner and the lamina, but not in the magnocellular subdivisions. In the dorsal cochlear nucleus (DCoN) the UBCs are found in all regions, but especially in layers 1 and 2 (Fig. 10 B). This mode of distribution indicates that the UBCs of the cochlear nucleus populate regions belonging to the "granule cell domain" as defined by Mugnaini et al. 1980. The dense calretinin immunostaining of the cochlear nerve fibers and the positivity of other coch-

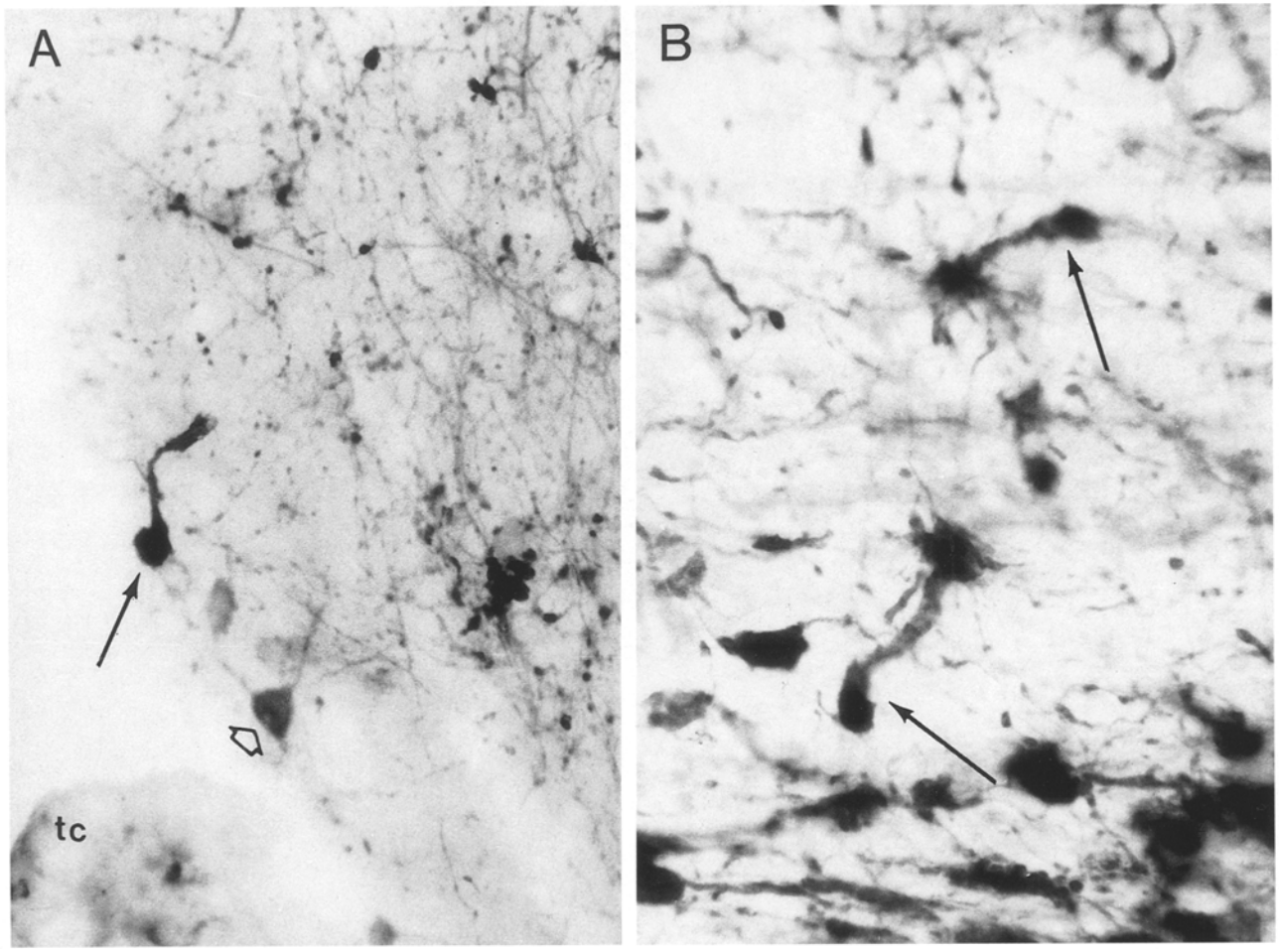

Fig. 10. Calretinin-immunoreactive elements in the cochlear nucleus, after osmium intensification. **A** The densely stained cell (*arrow*) is a UBC in the superficial granule cell domain; the less densely stained cell (*open arrowhead*) presumably represents a Golgi cell. *tc*, Tenia choroidea. × 500. **B** Two UBCs (*arrows*) in the superficial layers of the dorsal cochlear nucleus. × 1 000

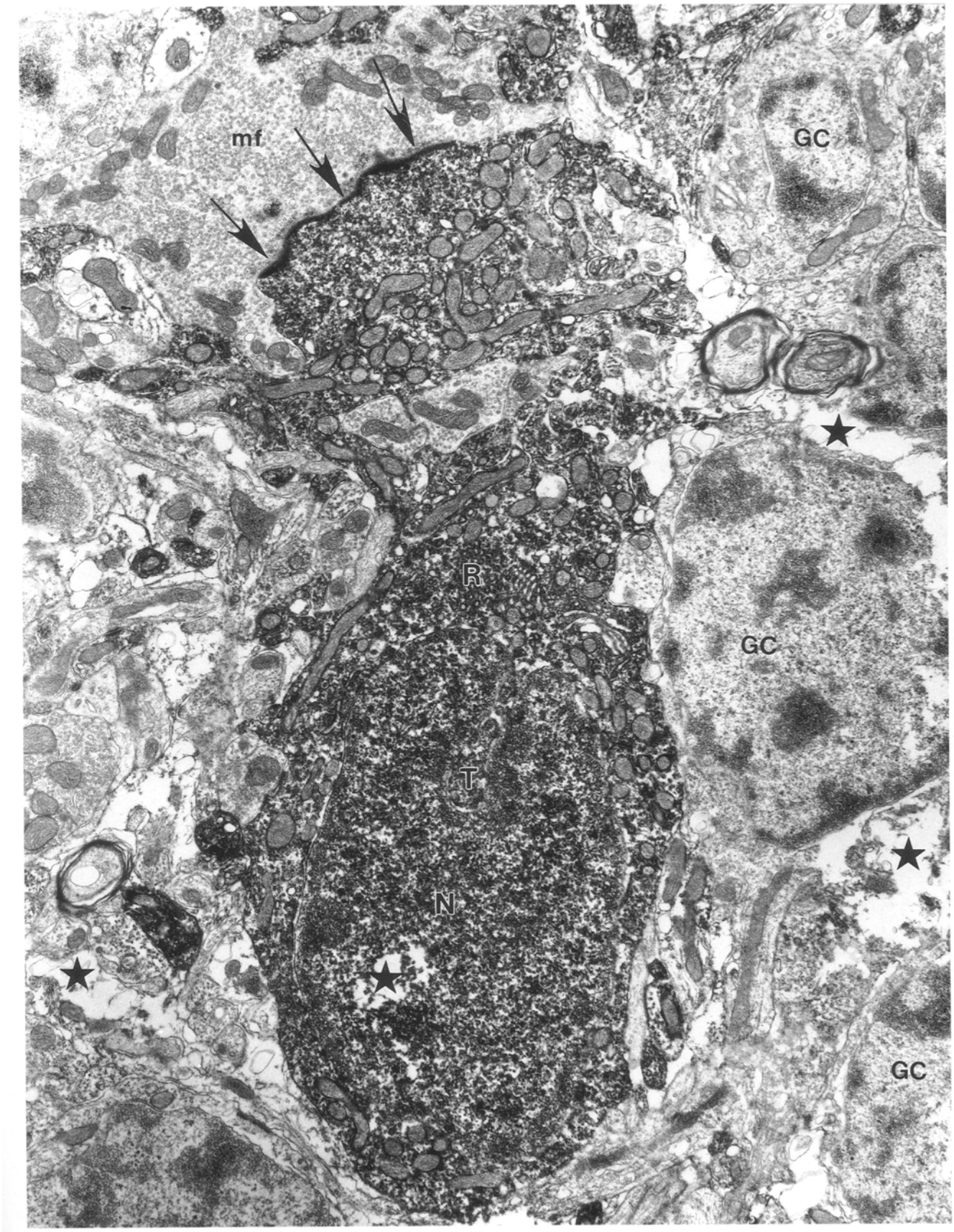

Fig. 11. Immuno-electron micrograph of a UBC stained with the pre-embedding protocol. The micrograph was obtained near the surface of the immunoreacted slice, as indicated by open areas in the tissue and over the cell nucleus (*stars*). The unipolar cell body has an irregular contour. The nucleus (*N*) shows a deep indentation (*T*) and the cytoplasm contains an array of ringlet subunits (*R*). The short dendrite forms an extensive synapse (*arrows*) with a calretinin-negative mossy fiber ending (*mf*). At the aspect opposite the synapse the dendrite contains numerous mitochondria. Granule cells (*GC*) are immunonegative. × 14 300

lear neurons has been described in detail elsewhere (Arai et. al. 1991; Winsky and Jacobowitz 1991; Résibois and Rogers 1992).

Immuno-electron microscopy

Immunopositive UBCs were easily identified in the re-embedded, immmunoreacted slices by transillumination of the Epon block under the light microscope. Because of the short DAB reaction time used in the pre-embedding protocol, only the most immunoreactive elements, i.e., the UBCs and the mossy fibers, are immunolabeled in these sections. Areas of the granular layer containing these elements were selected from the nodulus, the uvula or the flocculus, the blocks were trimmed, and ultrathin sections were cut on the ultramicrotome parallel to the surface of the re-embedded Vibratome slices, keeping record of the serial order of the grids on which the utrathin sections were mounted. In the electron microscope, the fine structure of UBCs and mossy rosettes usually appears partially obfuscated by electron-dense reaction product because of their strong immunoreactivities. Due to the omission of detergent permeabilization from the pre-embedding protocol, the only immunoreactive cellular elements are the UBCs and the mossy endings that are situated near the surface of the Vibratome-sliced tissue and have been cut open by the vibrating blade. Nevertheless, only some of the mossy fiber terminals that abut on the tissue surface are immunostained. This indicates that the selected folia contain calretinin-positive as well as calretinin-negative mossy fibers, because all the opened profiles have undoubtedly been exposed to the immunoreagents. In the UBCs closest to the surface of the immunoreacted slices, both the cell nucleus and the cytoplasm are immunostained (Fig. 11). Deeper in the tissue, however, only the perikaryon is immunostained and one notices that the nucleus contains little heterochromatin (Figs. 12, 13). The nuclear perimeter usually shows one or two indentations. In some of the cells the indentations are rather deep and give the nucleus a lobed appearance. The perikaryon contains few individual cisterns of granular endoplasmic reticulum, but has a large Golgi apparatus, coated vesicles, numerous mitochondria and large dense-core vesicles (Fig. 14). In many UBCs there is a peculiar, individual inclusion (Figs. 11, 12) that lacks a limiting membrane and is made up of ringlet subunits. In different cells, this inclusion occurred near the plasma membrane, the Golgi apparatus, the nucleus, within one of the cytoplasmic indentations protruding into the nuclear territory, and even in the dendritic trunk. Most of the UBC bodies carry thin appendages that terminate in the surrounding without forming any synaptic connections (Fig. 13). The large dendritic trunk seems to be out of proportion to the small size of the parent perikaryon, and, like the latter, is usually provided with non- synaptic appendages. The dendritic trunk terminates with a spray of twisted branchlets that enter one or two glomeruli (Figs. 11, 15–17). These glomeruli comprise immunonegative (Fig. 11) or immunopositive (Fig. 15) mossy fiber endings as well as a varying number of small, unstained dendritic profiles

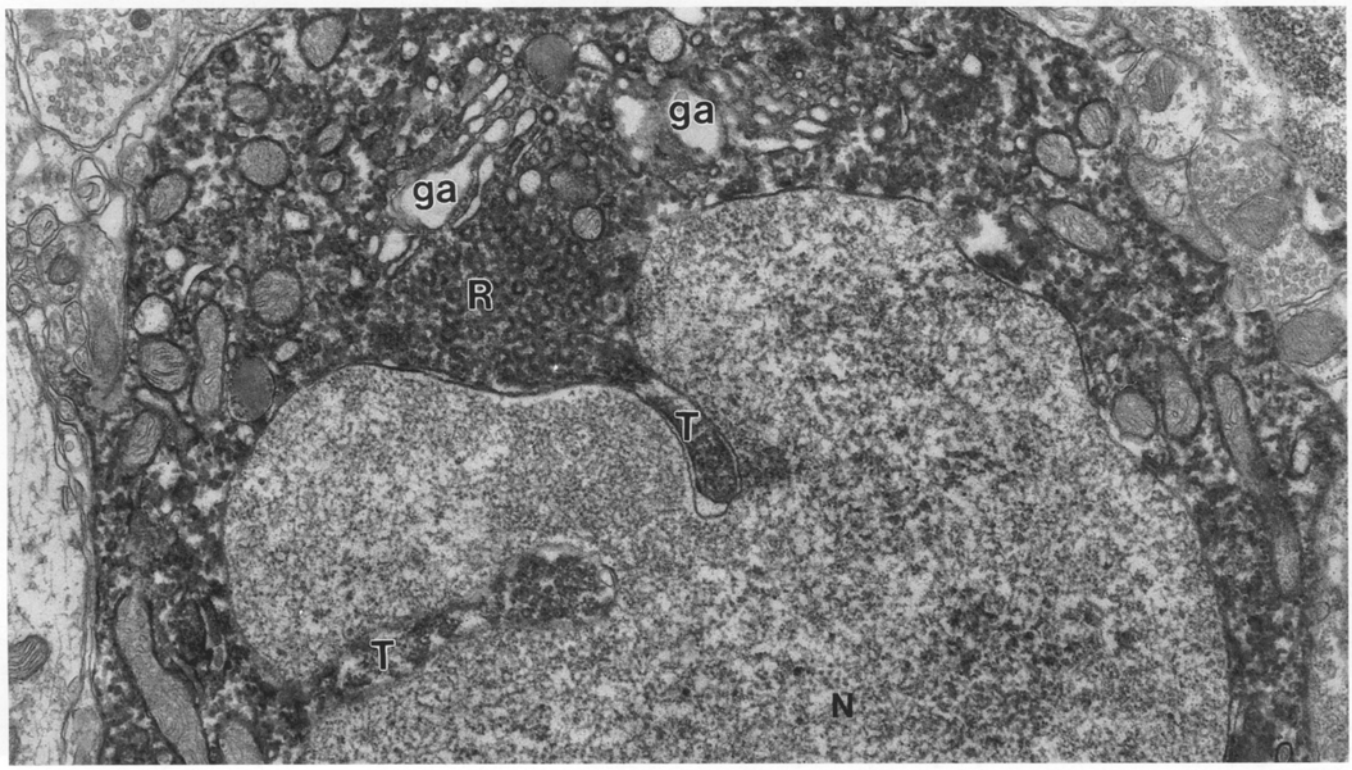

Fig. 12. Detail of UBC from the deep region of an immunoreacted slice. The nucleus (*N*) show indentations (*T*) and appears immunonegative. An assembly of ringlet subunits is indicated by *R*; *ga*, Golgi apparatus. × 18 150

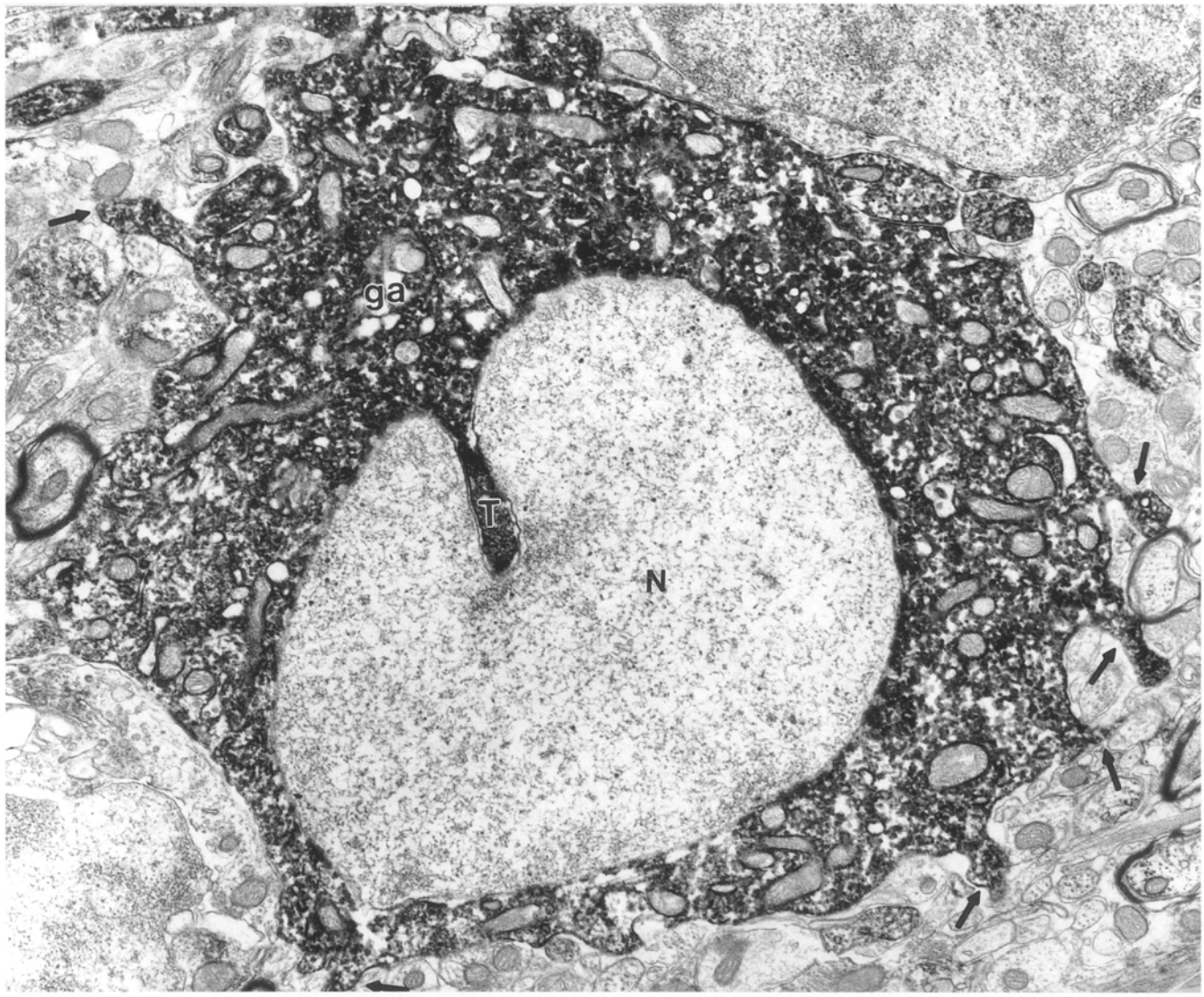

Fig. 13. Detail of UBC from the deep region of an immunoreacted slice. Somatic appendages (*short arrows*) are filled with immunoreaction product. *N*, nucleus; *T*, nuclear indentation occupied by immunoreactive cytoplasm; *ga*, Golgi apparatus. × 26 000

(Figs. 15, 16), which represent the digitiform tips of granule cell dendrites, an occasional large dendrite (data not shown), which may belong to the Golgi cell, and small boutons that contain pleomorphic vesicles and are interpreted as Golgi-cell axons (Figs. 15–17). The presumptive granule cell dendrites appear immunonegative because the pre-embedding protocol utilized in this study did not include detergent permeabilization and applied a short DAB reaction. These conditions are insufficient to reveal the weak immunoreactivity of the granule neurons. The shafts of the branchlets form synaptic junctions with the mossy rosettes (Figs. 15, 17), and, especially near the parent stem, they are also in synaptic contact with Golgi-like boutons (Fig. 15). The synaptic junction between branchlet and mossy ending consists of an unusually long, asymmetric active site (Fig. 11), a series of shorter, asymmetric active sites, or a combination of these two kinds of contact (Figs. 15, 17). All of these synapses are longer than those formed by mossy fiber and granule cell dendrites (thin arrows in Fig. 15). The synaptic junctions with the Golgi-like boutons usually consist of single short, symmetric active sites (arrowhead in Fig. 15), although long asymmetric contacts are occasionally observed.

We have also observed immunoreactive branchlets that contain a group of round synaptic vesicles crowded at the plasma membrane and facing a postsynaptic density, thus representing presynaptic profiles. Such an array is shown in Fig. 16, where the postsynaptic profile is an immunonegative dendrite which could represent the stem of a granule cell dendrite or a distal Golgi cell dendrite.

In all the immunostained branchlets, the postsynaptic cytoplasm immediately beneath the mossy fiber contact is occupied by dense precipitate (Figs. 11, 15). The interior of the postsynaptic branchlet contains numerous mitochondria and other membranous and cytoskeletal organelles (Figs. 11, 15–17). These organelles are poorly resolved in the immunoelectron micrographs taken near the surface of the Vibratome slices, where penetration of immunoreagents is maximal, but are more clearly seen in the immunoelectron micrographs taken from deeper re-

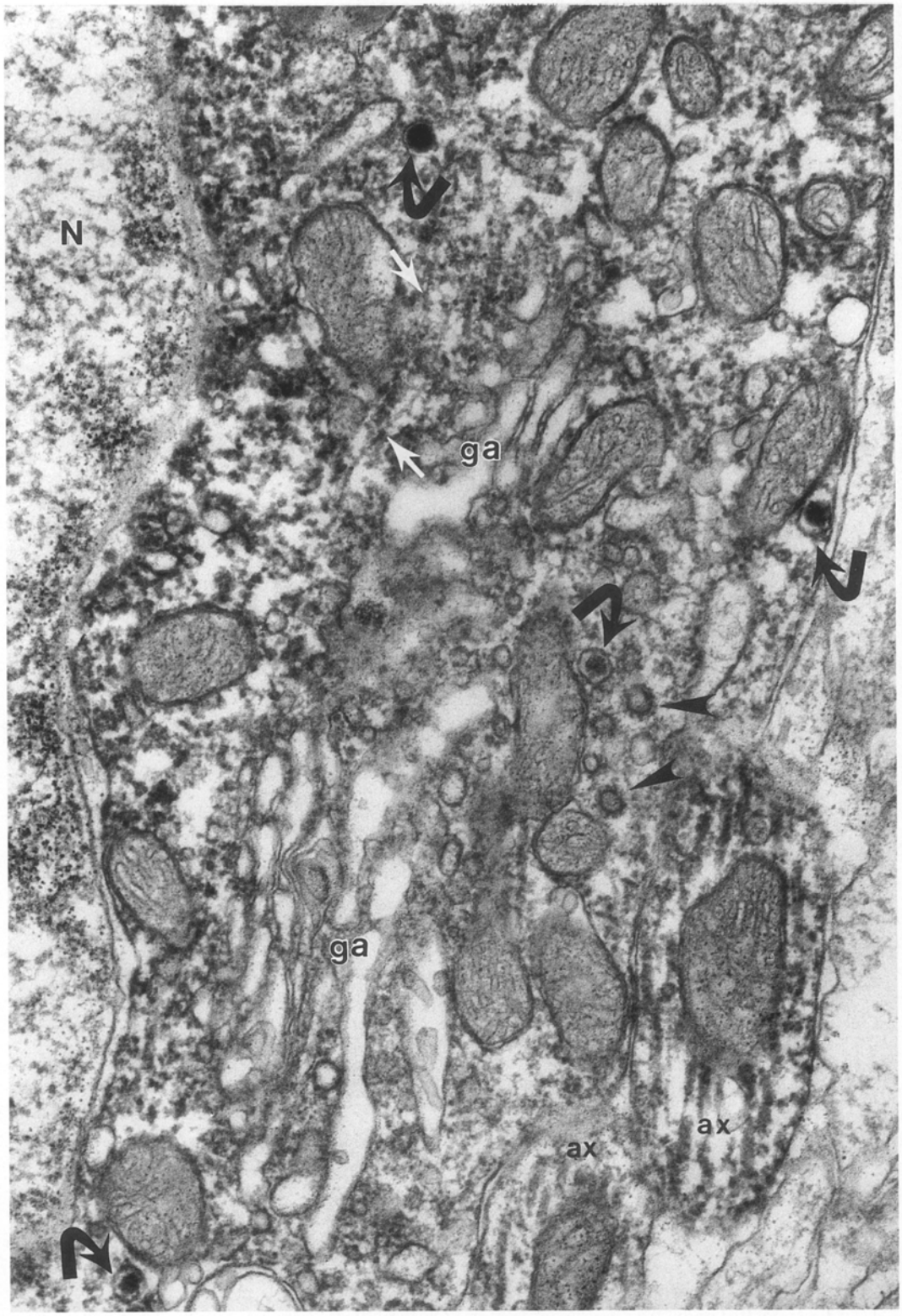

Fig. 14. Detail of a UBC showing part of the cell body and two nearby UBC axons (*ax*). *Curved arrows* indicate large dense-core vesicles; *N*, nucleus; *ga*, Golgi apparatus; *white arrows*, neurofilaments; *arrowheads*, coated vesicles. × 58 000

gions of the tissue, where there has been scarce penetration of immunoreagents (Fig. 17). In such micrographs, neurofilaments, microtubules, tubules of smooth endoplasmic reticulum, polyribosomes, and dense-core vesicles are evident inside the branchlets. Neurofilaments are absent in the immunoreactive cytoplasm immediately beneath the mossy fiber synapse. Numerous non-synaptic appendages emanate from the sides of the branchlets that are not engaged in the synapse. These appendages are often free of mitochondria and other organelles and re-

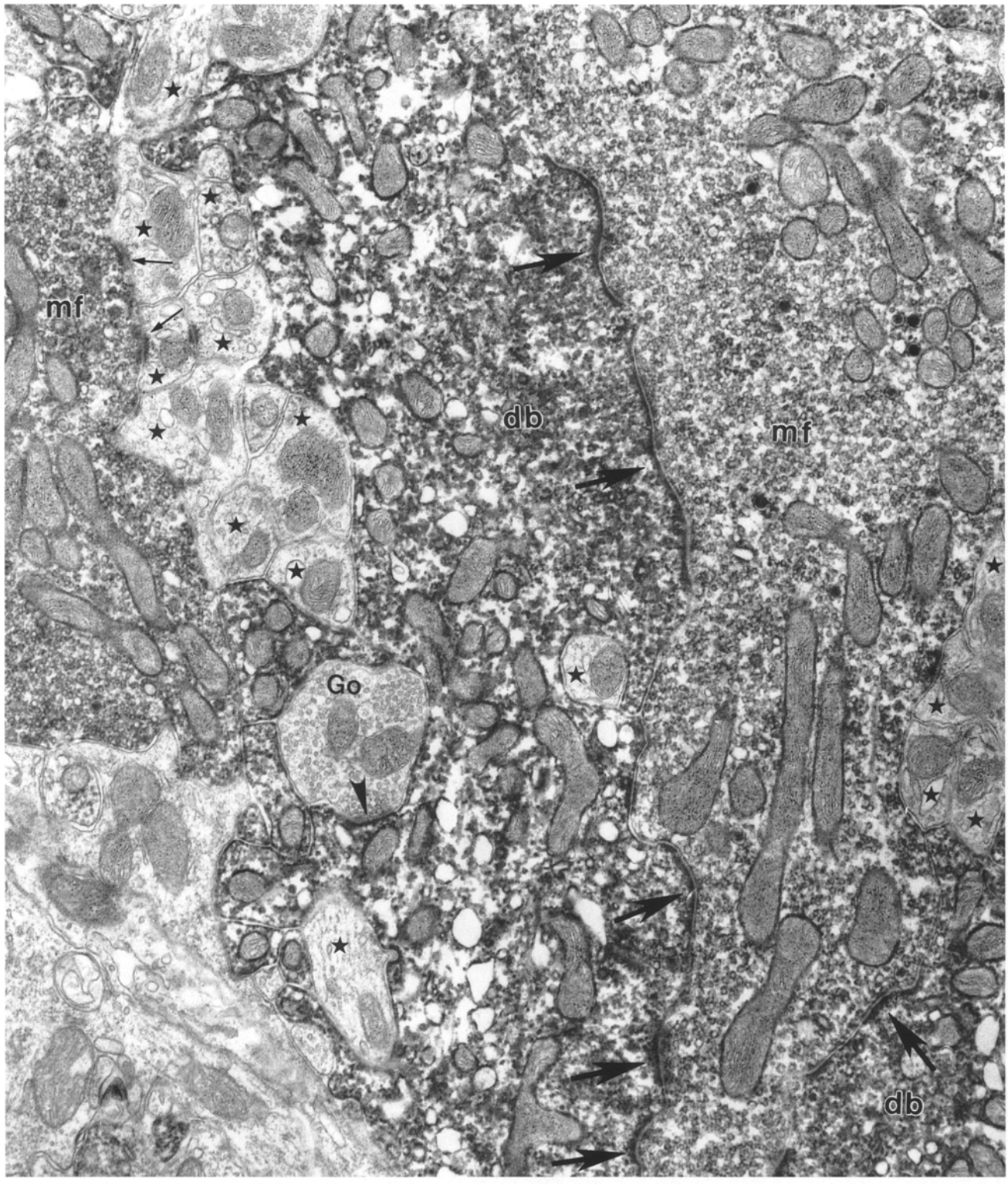

Fig. 15. Detail from a glomerulus. The synaptic array includes: immunoreactive dendritic branchlets of a UBC (*db*); parts of an immunoreactive mossy fiber rosette (*mf*), which are filled with small, clear synaptic vesicles, large, dense-core vesicles and mitochondria; several immunonegative granule cell dendrites (*stars*); and an axon terminal (*Go*), tentatively identified as a Golgi cell bouton, that is filled with pleomorphic synaptic vesicles and forms a symmetric synapse (*arrowhead*) with one of the UBC branchlets. The mossy rosette forms extensive asymmetric synapses with the UBC branchlets (*thick arrows*), and small asymmetric synapses (*thin arrows*) with granule cell dendrites. × 25 400

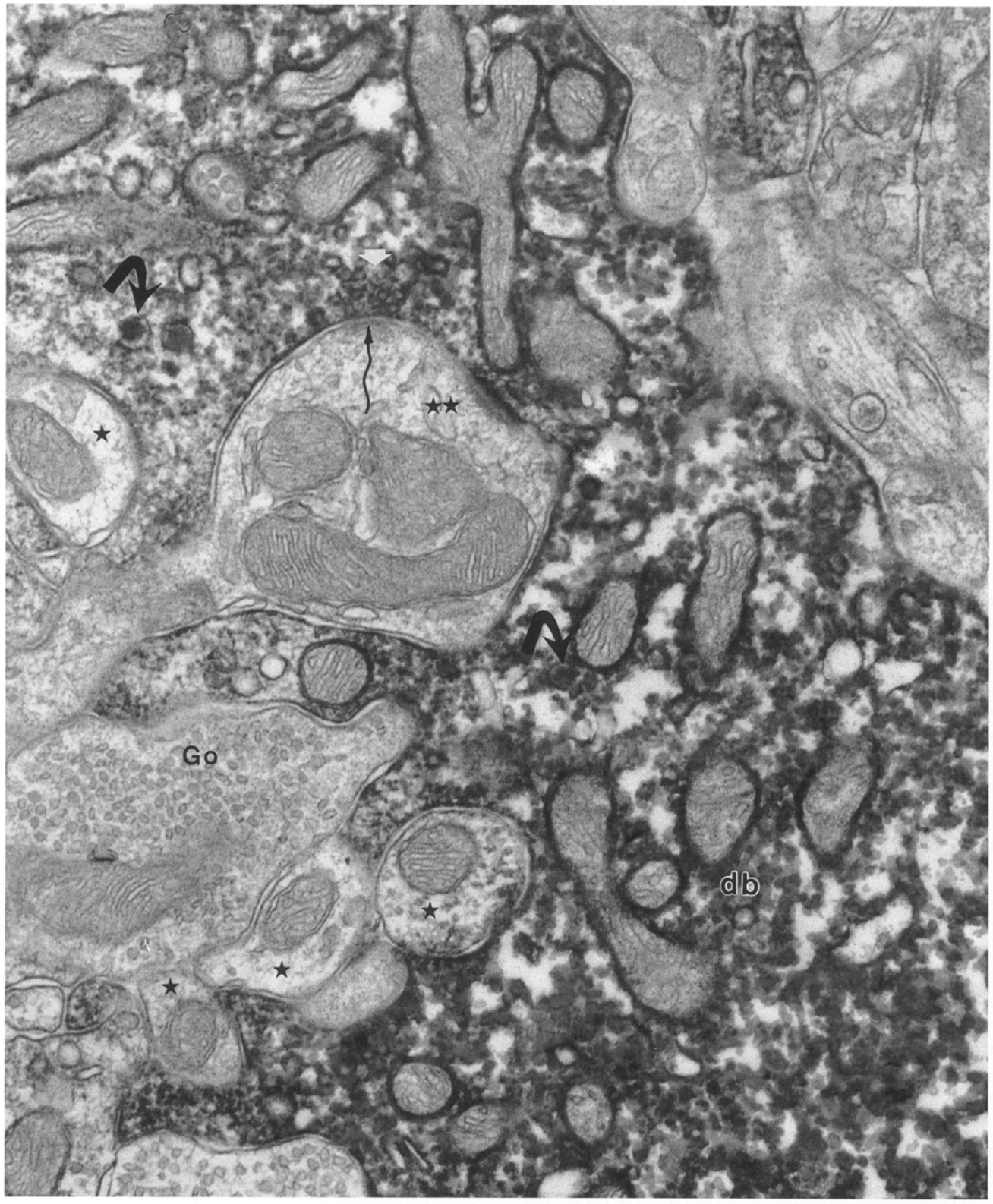

Fig. 16. Detail from a glomerulus. The micrograph includes portion of an immunoreactive dendritic branchlet (*db*) of a UBC, immunonegative granule cell dendrites (*single stars*), a Golgi-like bouton (*Go*), and an immunonegative dendrite (*double star*). The latter receives a synapse (*kinky arrow*) from the dendritic branchlet in which a cluster of small clear vesicles is evident (*white arrow*). *Curved arrows* indicate large dense-core vesicles. × 54 000

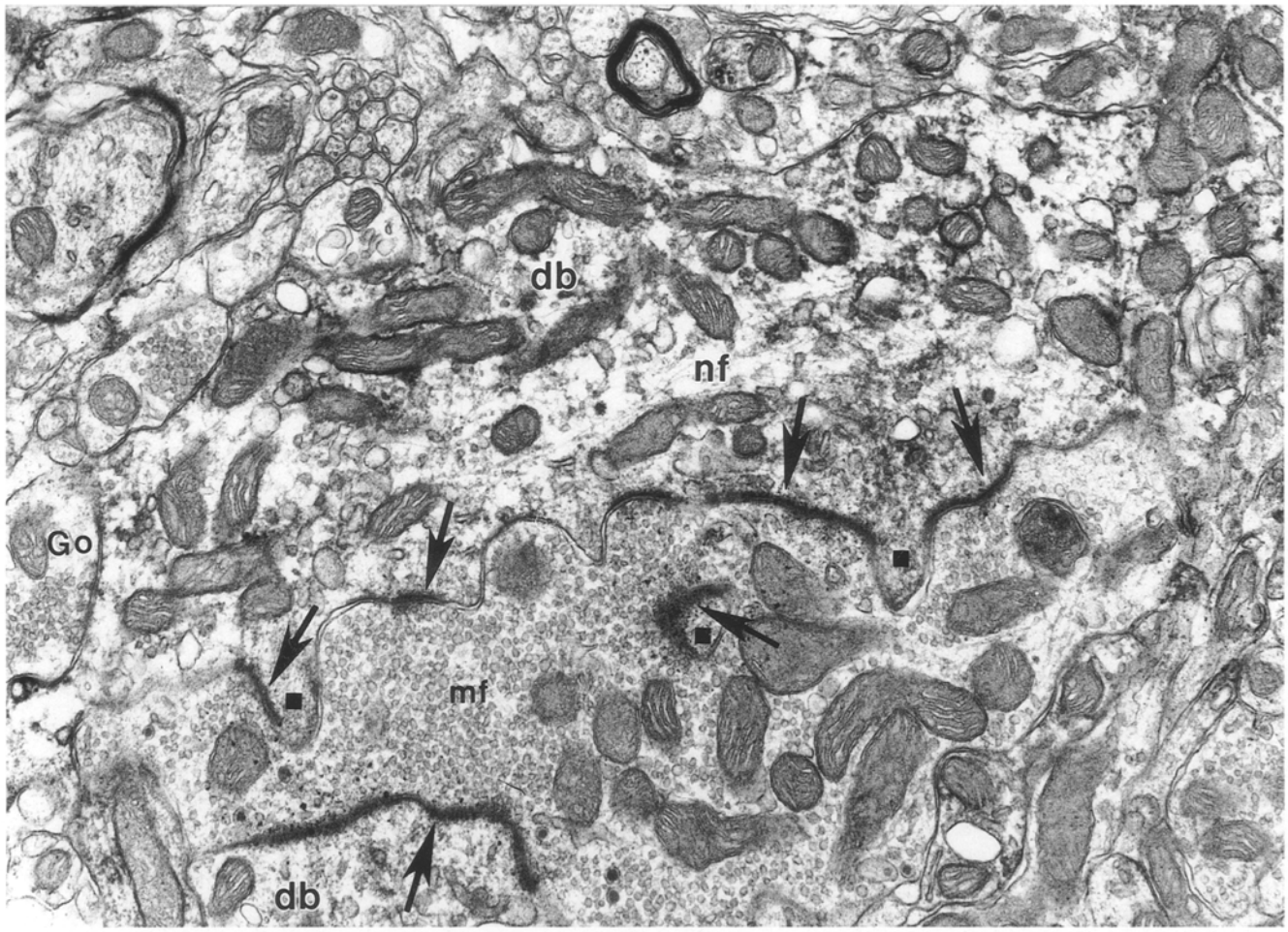

Fig. 17. Branchlet organelles. This electron micrograph was obtained from the deep portion of an immunoreacted slice and includes two dendritic UBC branchlets (*db*) that are weakly immunolabeled. The branchlets are in synaptic contact (*arrows*) with an immunonegative mossy rosette (*mf*). The scarce immunoreaction product in the branchlet allows better discrimination of the dendritic organelles, which include numerous mitochondria, tubular and vesicular elements of smooth endoplasmic reticulum, and two large, dense-core vesicles. In addition, there are neurofilaments (*nf*), microtubules and polyribosomes that are not easily recognized at this magnification. *Black squares* mark parts of the branchlet invaginating the mossy rosette. *Go*, Golgi-like bouton. × 26 500

semble those emanating from the cell body and dendritic trunk. Some protrusions of the branchlets may invaginate the mossy fiber ending (Fig. 17), and some protrusions of the mossy fiber ending may invaginate the spiny branchlet. Some of the UBCs are contacted by mossy fiber endings directly on the soma or very near (Fig. 11) the cell body. In these cases large accumulations of mitochondria are situated in the vicinity of the synapse. The somata of some of the UBCs (not illustrated) emit, in addition to a large dendritic stem, a number of short, mitochondria rich dendritic branchlets that establish synaptic contact with mossy fiber endings. The mossy fiber endings engaged in synaptic contact with the dendritic branchlets of the UBCs vary considerably in shape, size and internal fine structure. Some of them are large (Figs. 11, 15), and have the shape of the classical rosettes usually encountered inside glomeruli whose dendritic components are exclusively provided by granule cells. Other mossy endings, on the contrary, are thinner (Fig. 17) or outright slender, or even consist of varicosities connected by thin stalks. Some of the mossy endings are filled with small, clear and round synaptic vesicles (Fig. 11). Others, in addition, contain a considerable proportion of large dense-core vesicles (Fig. 15).

Discussion

In this study we have extended previous observations on the cerebellar localization of calretinin-like immunoreactivity in the rat and we have contributed new knowledge on the ultrastructural features of the unipolar brush cells (UBCs), which are most densely stained by calretinin antiserum. The pattern of calretinin immunostaining in the rat cerebellum had been described previously, albeit only briefly. Our observations confirm the general descriptions outlined in the rat hindbrain atlases of calretinin immunoreactivity recently published by Rogers (1989), Arai et al. (1991), and Résibois and Rogers (1992). Moreover, we highlight the characteristic, strong immunostaining of the UBCs, the topographic variations in immunostaining of granule cells and parallel fibers, the

weak immunostaining of climbing fibers, and the relatively dense immunostaining of neurons and axonal plexuses in the cerebellar nuclei, four points that were not firmly established or were not recognized by the previous authors. Furthermore, because the UBCs usually are the only neurons in the cerebellar cortex that show strong labeling with our calretinin antibody, it was possible not only to make a clear map of the distribution of these cells, but also to analyze their ultrastructure utilizing a pre-embedding protocol for immuno-electron microscopy. The UBCs will be discussed first because of their prominent immunostaining and novelty. Comments on the other cerebellar calretinin-positive elements will follow.

Light microscopic identification of the unipolar brush cells

Calretinin is an excellent marker for the UBCs throughout the cerebellar cortex. We have confirmed the data from Golgi impregnated sections indicating that these cells are intermediate in size between granule and Golgi cells, and differ from both these other well-known cerebellar neurons in being usually monopolar, their single dendrites terminating in brush-like formations. In a separate paper, Harris et al. (1993) have also shown that UBCs stand out from neurons of the granular layer because they are densely stained by antibodies to the high-molecular-weight neurofilament protein. The Golgi method and calretinin immunostaining clearly show that UBCs are distinct from the multipolar Golgi cells. Golgi cell axons ramify near the parent cell bodies forming a dense plexus (Cajal 1911). After intracellular dye injections in thin slices, UBC axons have been shown to form individual branches in the granular layer, provided with mossy-like endings (Rossi et al. 1994). Furthermore, in Golgi and immunostained sections, UBC axons were occasionally seen to enter the white matter (Mugnaini and Floris 1994, and our Fig. 2 B), presumably to form distant projections. Therefore, while the multipolar Golgi cells are classical examples of local circuit (Golgi type II) neurons, the UBCs may represent a new type of cortical efferent neuron with recurrent collaterals (Golgi type I neuron). Unfortunately, both Golgi impregnations and immunostaining with either calretinin (this study) or neurofilament antibody (Harris et al. 1993) have proved inadequate to reveal the UBC axon in its entirety. The Golgi-Hortega method utilized by Mugnaini and Floris (1994) impregnated the UBC axon for only a short distance. The presence of calretinin and neurofilament-positive cerebellar afferents made it impossible to follow the UBCs to their termini in the immunostained sections. The precise projections and synaptic targets of the UBCs, therefore, remain to be ascertained with other labeling procedures, such as intracellular dye injections and retrograde transport methods.

In spite of their distinctive features, the UBCs have been recognized only recently as particular entities. Mugnaini and Floris (1994) have presented arguments in favor of the notion that the UBCs correspond to cells previously termed pale cells (Altman and Bayer 1977; Sturrock 1990), secretogranin II positive small cells (Cozzi et al. 1989), chromogranin A positive cells (Munoz 1990), calretinin-positive small cells (Arai et al. 1991; Winsky and Jacobowitz 1991; Floris et al. 1992, Résibois and Rogers 1992; Braak and Braak 1993). Harris et al. (1993) have shown that UBCs rich in neurofilament proteins correspond to the Rat-302 cells (Hockfield 1987). The calretinin-positive UBCs possess a peculiar fine structure. Their features and distribution are similar to those of the cells in the rat cerebellum revealed by antibodies to the neurofilament proteins (Harris et al., 1993). We presume, therefore, that calretinin and neurofilament antibodies reveal members of the same cell population.

Fine structure and synaptology of the unipolar brush cell

The dendritic trunk and terminal branchlets, and often also the cell body of the UBC emit a substantial number of non-synaptic appendages, the function of which is unclear. The appendages are free of neurofilaments (Harris et al. 1993), but they are densely calretinin-positive, which suggests they may become permeated by calcium. A re-evaluation of Golgi cells and granule cells by standard electron microscopy (Mugnaini et al. 1994) indicates that these neurons have rather smooth cell bodies. Golgi cell dendrites bear few spines, which usually appear postsynaptic to varicosities of granule axons. Granule cell dendrites have a smooth contour and end with tips that are often slightly larger and contain more abundant cytoplasmic organelles than the non-synaptic appendages of the UBCs, and are postsynaptic to mossy rosettes and Golgi cell axons. Dendrites with ultrastructural features closely resembling those of UBCs have previously been described by Hámori and Szentágothai (1966), Eccles et al. (1967), and Mugnaini (1972) in the cat cerebellum, by Palay and Chan-Palay (1974) in the rat cerebellum, and by Mugnaini et al. (1980) in the granule cell domain of the cochlear nuclei of various mammals. These dendrites, however, were interpreted as belonging to large and small multipolar Golgi cells, an identification that is in contrast with the results of the present study.

The UBC perikarya contain higher concentrations of mitochondria and Golgi lamellae than expected in small neurons and possess a peculiar cytoplasmic inclusion that consists of ringlet subunits. The surrounding, electron-dense immunoprecipitate prevented a detailed analysis of the subunits. In standard electron micrographs (Mugnaini et al., 1994), these appear as non-membranous entities. Their chemical composition remains to be ascertained. In the rat, cytoplasmic assemblies of ringlet subunits were absent from other cerebellar neurons, and they can be used as a marker for the identification of the UBCs (see also Harris et al., 1993). A similar inclusion was first described by Mugnaini (1972, his Fig. 67) in the cat cerebellum, but he thought, presumably erroneously, that it occurred in a Golgi cell.

UBCs contain numerous large, dense-core vesicles, not only in the cell body, but also in the dendritic trunk, the branchlets and the appendages. Large, dense-core

vesicles are usually associated with one or more neuropeptides, and with proteins of the chromogranin-secretogranin family (reviewed by DeCamilli and Jahn 1990; Winkler and Fischjer-Colbrie 1992). This supports the suggestion that calretinin-positive UBCs are identical to the secretogranin II (Cozzi et al. 1989) and the chromogranin A (Munoz 1990) -positive small cells, which they closely resemble in shape. Double-labeling immunofluorescence experiments are in progress to make a definitive assessment of this identification. In other kinds of neuron it has been suggested that large, dense-core vesicles can be released independently from junctional presynaptic specializations (DeCamilli and Jahn 1990). Their abundance in the UBCs makes it highly probable that the putative neuropeptide is involved in a paracrine, or perhaps even an autocrine, mechanism. They also show clusters of small, clear vesicles, crowded at synaptic junctions formed with dendrites, that may belong to Golgi cells and granule cells. The presence in the UBC branchlets of presynaptic clusters of clear, small vesicles indicates that these peculiar cells belong to the category of neurons that are provided with an axon but possess dendrites that are both pre- and postsynaptic. The shape of these small vesicles could not be defined with certainty in our immunostained ultrathin sections. However, Mugnaini et al. (1994) have shown by standard electron microscopy that identically situated small vesicle clusters of the UBCs are definitely round in shape. This observation carries an interesting corollary: namely, round vesicles are usually associated with excitatory neurotransmitters. If the UBC carries the same type of vesicle also in the axon, a likely assumption in view of the present knowledge about neural cell biology (Peters et al. 1991), it would appear probable that the UBCs have an excitatory effect on their targets. This hypothesis is supported by the finding that the UBCs are both GABA- and glycine-negative (Mugnaini et al. 1994), contrary to the Golgi cells, which release from their terminals both of these inhibitory neurotrasmitters in a calcium-dependent manner (Ottersen et al. 1988).

The most notable feature of the UBC is that it receives synapses primarily at its dendritic brush, which participates in only one or two glomeruli. Few neurons in the central nervous system are so clearly devoid of converging excitatory inputs. The synaptology of the UBC, however, indicates that it receives an unusually extensive synapse from the mossy fiber rosette with which its dendritic tip articulates. The high concentrations of mitochondria in the branchlets facing these unusually large synaptic junctions suggest that the synapse is coupled to a region of high metabolic activity, possibly involved with supplying energy for ionic pumps. The strong calretinin immunoreactivity of the UBCs may also be related to their unusually extensive synapses with the mossy fiber rosettes. Calretinin may provide a fast buffering action for calcium entering at the synapse from the extracellular space and also for calcium released from intracellular stores.

The symmetrical synapses that the UBC dendrite forms with small boutons containing pleomorphic synaptic vesicles suggest that the activity of these cells is inhibitorily modulated by the Golgi cells. Whether Purkinje recurrent collaterals also synapse on the brush cells is now being investigated by immuno-electron microscopy with antibodies to Purkinje cell-specific markers.

It should be noted that some of the peculiar synaptic features discussed here have previously been described in the literature. Chan-Palay and Palay (1971) and Palay and Chan-Palay (1974), in the rat cerebellum, and Mugnaini et al. (1980), in the granule cell domain of the cochlear nuclei, have described a special axo-somatic arrangement that the Palays distinctively termed "en marron synapse". Hamori and Szentágothai (1966) and Mugnaini (1972) emphasized the multiple synaptic contacts between certain dendrites of the cerebellar granular layer and the mossy fibers. All the above synaptic contacts were attributed to Golgi cells. We show here, instead, that these synaptic features are typical of UBCs (see also Harris et al. 1993; Mugnaini et al. 1994). Furthermore, dendritic assemblies of clear synaptic vesicles in cerebellar neurons have been observed by Sotelo (1977) in rats after neonatal X-irradiation, but these assemblies were attributed to Golgi cells, which he interpreted as rearranging their intracellular traffic of organelles as a consequence of the altered circuitry. Whether the dendritic assemblies of clear vesicles in the irradiated cerebellum are partly attributable to UBCs needs to be assessed with the aid of immunoelectron microscopy.

Distribution of the unipolar brush cells

Some knowledge about variation in the frequency of occurrence of the UBCs across the cerebellar folia of mouse, rat, cat, and rhesus monkey was provided by Mugnaini and Floris (1994), on the basis of impregnations with a variant of the Golgi-Del Rio Hortega procedure. They reported that UBCs in all species were prevalent in the flocculo-nodular lobe and other folia belonging to the vestibulo-cerebellum, occurred at moderate density in other vermal folia, and were rarely seen in the cerebellar hemispheres. Although this Golgi protocol impregnates a much larger proportion of neurons than the classic Golgi rapid method, it still represents an inadequate procedure for mapping distinct neuron types. In spite of this, the observations of these authors are supported by studies in the rat cerebellum with antibodies to the high-molecular-weight neurofilament protein (Harris et al. 1993), to secretogranin II (Cozzi et al. 1989), to calretinin, as presented here and by Rogers (1989), Arai et al. (1991), Floris et al. (1992), and Résibois and Rogers (1992), and by data on the human brain reported by Munoz (1990) with antibody to chromogranin A (Munoz 1990) and by Floris et al. (1992) and Braak and Braak (1993) with different antisera to calretinin.

The UBCs' distribution maps presented in our Figs. 5 and 6 may be somewhat imprecise, insofar as they are based on the assumptions that all UBCs are calretinin-positive and that all immunostained cell bodies 9–12 μ m in diameter which are not demonstrably multipolar are, in fact, UBCs. Yet these maps undoubtedly emphasize

the paucity of UBCs in the lateral folia of crus II and the dorsal paraflocculus, their moderate density in the intermediate cortex, the abundance in lobules IXd, X and the flocculus, and presence of relative hot spots in the most lateral region of crus I and at the flocculus /paraflocculus transition. This mode of distribution suggests that the UBCs migrate into the cortex in a specific topographic pattern and may be chemotropically guided by special groups of mossy fibers. This migration may only partly follow the known parasagittal and rostrocaudal boundaries (reviewed by Smeyne et al. 1991; Sotelo and Wassef 1991; Oberdick et al. 1992), and a study of the ontogeny of UBCs may reveal novel aspects of cerebellar compartmentation.

Because the foliation pattern of the cerebellar cortex may vary in fine details (Welker 1987) and individual variations in the distribution of UBCs, albeit small, may exist, quantitative topographic mapping of the UBCs requires a separate and careful study. Two brief reports (Floris et al. 1992; Braak and Braak 1993) and an extensive study (Diño, Jacobowitz and Mugnaini, in preparation) show that the UBCs remain the most calretinin immunoreactive elements across all mammals so far examined, including human beings. Unfortunately, however, calretinin immunoreactivity in the cerebellum of non-mammalian species is much more widespread than in the rat cerebellum (Rogers 1989), and may not be useful to uncover possible analogues of the mammalian UBCs across vertebrates.

In this paper, we wish to stress a few general points concerning the density of UBCs, in particular that they are very concentrated in the vestibulo-cerebellum, and that they may outnumber Golgi cells by a large factor in the flocculo-nodular lobe. Lange (1974) and Brodal and Drabloes (1963), Palkovits et al. (1971) among others, have previously noted that the density of presumed Golgi cells is much higher in these folia than in other regions of the cerebellar cortex. Because of the confusion between UBCs, Golgi cells and astrocytes in the older literature, the true concentration of Golgi cells across the cerebellum needs also to be quantitatively re-evaluated. Since the demonstrations that antibodies to glutamate decarboxylase (Oertel et al. 1981; Mugnaini and Oertel 1985) and GABA (Storm-Mathisen et al. 1983; Ottersen et al. 1988) stain all Golgi cells, these antibodies have remained an ideal differential cell-class marker for the Golgi neurons.

The presence of UBCs in the superficial granular layer of the VCoN and in the DCoN was not unexpected (see also Winsky and Jacobowitz 1991; Floris et al. 1992; Résibois and Rogers 1992), in view of the fact that the cochlear nuclei contain a neuronal microcircuit analogous to that of the cerebellar cortex. This microcircuit includes a system of mossy fibers impinging upon granule and Golgi cells, and a system of parallel fibers innervating stellate and cartwheel cells (the latter being Purkinje cell-like neurons) in the superficial layers of the DCoN (reviewed by Mugnaini and Morgan 1987; Berrebi and Mugnaini 1991, 1993). The UBCs represent yet another type of neuron shared by the cochlear nuclei and the vestibulo-cerebellum. Advances in the knowledge about this neglected type of cell, therefore, will help clarify both vestibular and auditory mechanisms.

Immunostaining of mossy fibers

Our study confirms the existence of mossy fibers that are intensely immunopositive for calretinin in ventral cerebellar folia, as described by Arai et al. (1991) and Résibois and Rogers (1992). In addition, we have observed the presence of weakly stained glomeruli in most folia. It remains to be established to what extent calretinin antiserum can be used to label specific populations of mossy endings. The strongly immunoreactive mossy rosettes, which are distributed in folia with the highest concentration of UBCs, must originate, at least for the most part, from the vestibular ganglion, the vestibular nuclei and the nucleus prepositus hypoglossi, because these centers contain strongly stained efferent neurons that innervate the ventral folia. The calretinin-positive mossy fiber shown in Fig. 15 presumably belongs to this category. Weakly immunostained mossy rosettes may originate from nuclei provided with weakly calretinin-positive neurons, such as external cuneate nucleus, trigeminal sensory nuclei, pontine gray, and reticular formation (Arai et al. 1991; Résibois and Rogers 1992), which are known to provide mossy fibers to vermal and hemispheral folia. The granular layer collaterals of the UBC axons (Rossi et al. 1994) may form yet another population of immunoreactive mossy endings; because the UBC axon is much less distinctly stained than the cell body and the dendrite, it is uncertain whether these endings fall within the densely or weakly immunostained categories.

Immunostaining of granule cells and parallel fibers

We have demonstrated here that calretinin immunoreactivity of granule neurons shows topographic differences in the same specimen. As shown in Fig. 1 and 2, a sharp drop in granule cell immunoreactivity in the rodent cerebellum distinctly coincides with a sharp increase in the density of UBCs. The large areas of apposition between mossy fibers and UBC branchlets may imply that a smaller proportion of the synaptic area of mossy endings is available for granule cell dendrites, and granule cells might thus be less stimulated by mossy fiber inputs. If calretinin expression were transsynaptically regulated by mossy fiber input, this situation would result in less staining of the granule cells in regions of high UBC density. Alternatively, calretinin expression in granule cells of the vestibulo-cerebellum may be down-regulated by unknown factors.

Immunostaining of climbing fibers

The terminal arborizations of climbing fibers in the ceberellar cortex have been difficult to recognize with methods other than the Golgi procedure. In the cerebellum of the rat and other mammals, they have been more recently

revealed with antibody to corticotropin releasing factor (CRF) (reviewed by Mugnaini and Nelson 1989; King et al. 1992). The studies of Rogers (1989 a) in the chicken and the present investigation indicate that calretinin can be added to the list of endogenous climbing fiber markers. Unfortunately, however, calretinin immunostaining of climbing fibers is much less dense in the rodent than in the avian cerebellum, and immunostaining of rat climbing fibers by calretinin antibodies may remain of little value, unless powerful enhancement procedures for the immunoreaction are introduced into the protocol.

Immunostaining of cerebellar nuclei neurons

In this paper we have demonstrated that many small neurons in the cerebellar nuclei show calretinin-like immunoreactivity. Recent studies have indicated that numerous small neurons in the cerebellar nuclei are inhibitory, and that most of them project to the inferior olive (reviewed by Nelson and Mugnaini 1991). Thus, antiserum to calretinin represents an additional, potentially useful cell marker for studying the population of small neurons in the cerebellar nuclei, in addition to glutamate decarboxylase and GABA. Double-labeling experiments, with calretinin and neurotransmitter antibodies, and with calretinin and HRP retrogradely transported from the inferior olive may ascertain whether the calretinin-positive small neurons correspond, at least in part, to cerebellar neurons that inhibit climbing fiber activity.

To summarize, the UBCs are uniquely shaped neurons of the cerebellar granular layer that can advantageously be revealed by antiserum to calretinin. The form of the UBCs remains highly constant across all lobules, and their peculiar ultrastructure suggests that these neurons are endowed with unique biological and synaptic properties. Characterization of the features of the UBCs in Golgi-impregnated material and elucidation of their ultrastructure by immunocytochemistry with antibodies to calretinin and neurofilament proteins has allowed the establishment of firm criteria for their identification. As shown in a separate study (Mugnaini et al. 1994), these observations make it possible to identifiy these unique cerebellar neurons in standard electron micrographs, where their ultrastructure can be observed unencumbered by the electron-dense immunoprecipitate.

This study confirms that UBCs are primarily present in the nodulo-floccular lobe of the rat cerebellum, occur at relatively high density in the lingula, at moderate density in the vermis and the narrow intermediate cortex, while they are rare in most of the lateral hemispheral folia. Although the UBC-abundant regions of the cerebellar cortex include all the territories of distribution of primary and secondary vestibular fibers (Korte and Mugnaini 1979; Ojima et al. 1989; Barmack et al. 1992 a, c, 1993), the UBC maps shown in Figs. 5 and 6, indicate that these neurons are also present in folia that are presumably devoid of substantial vestibular input, although they populate the hemispheres only minimally. Thus, the UBCs seem to be involved in neuronal circuits related to posture, and the execution and coordination of motor events, rather than in the initiation of movements. Recent work indicates that spreading of the UBCs over the cerebellar cortex is greater in carnivores and primates than in rodents; nevertheless, their preferential distribution in the vestibulo-cerebellum seems to be largely maintained in different mammals, including macaque monkeys and human beings (Braak and Braak 1993; Diño, Jacobowitz and Mugnaini, in preparation). Although the origin of the mossy fibers synapsing on the UBCs is not yet firmly established, input sources probably include both vestibular ganglion cells and neurons in the vestibular nuclei and the nucleus prepositus hypoglossi. Because these afferents may use glutamate or acetylcholine as neurotransmitters, immunolocalization studies of their receptors may aid in the classification of the UBC synapses. Moreover, the varying features of the organelles contained within the mossy endings synapsing with the UBCs may also be correlated with their multiple sources of afferents.

The very high density of UBCs in the nodulus and the flocculus facilitates the study of the electrical properties of this peculiar type of neuron and its giant synapse in the slice preparation (Rossi et al. 1994) and may ultimately also aid in elucidating its functional role in the cerebellar circuitry.

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