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Authors

Ribak, Charles E
Woody, Charles D
Nahvi, Mahmood J
et al.

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Ultrastructural Identification of Physiologically Recorded Neurons in the Cat Cerebellum

CHARLES E. RIBAK, CHARLES D. WOODY, MAHMOOD J. NAHVI,
AND ERIC TZEBELIKOS¹

Department of Anatomy, University of California, Irvine, California 92717, and Departments of Anatomy and Psychiatry, University of California, Los Angeles, California 90024

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Neurons from the cerebellar cortex of cats were examined in electron microscopic preparations after intracellular recording and pressure injections of horseradish peroxidase (HRP). Neurons that contained HRP reaction product within dendrites were identified as either Purkinje or Golgi II cells. This identification was based on specific ultrastructural criteria that included the presence of synapses on the surfaces of dendritic shafts or spines, the identification of the presynaptic component of these synapses, and the presence of certain visible intracellular organelles. In addition, we examined specimens that contained two types of labeled dendrites after a single HRP injection. These dendrites were identified as arising from Purkinje and Golgi II cells and were shown to interdigitate with each other in a dendritic glomerulus. Dendritic appendages or sheet-like spines emanated from the Purkinje cell dendrite and sent small finger-like protrusions that surrounded and invaginated the Golgi II cell dendrite. In this glomerulus, the dendrites were shown to approach each other, and preliminary results suggest the presence of a gap junction at this site of direct apposition. This finding supports physiologic data which suggest electrical coupling between Purkinje and Golgi II cells. In addition, the results of this study demonstrate the usefulness of combining intracellular electrophysiology with HRP staining for the ultrastructural identification of recorded neurons.

INTRODUCTION

The advantages of observing physiologically recorded horseradish peroxidase (HRP)-filled neurons in the electron microscope were not

Abbreviations: HRP—horseradish peroxidase; GABA, γ -aminobutyric acid.

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exploited until recently (3, 4, 7, 20). In one of those studies, Christensen and Ebner (3) described in ultrastructural preparations the types of synaptic contacts made with the different parts of HRP-injected neurons in opossum cerebral cortex. By classifying these contacts into either asymmetric or symmetric types and by determining their distribution upon the surfaces of labeled cells, those workers could distinguish different types of recorded neurons.

In the preceding article (11), patterns of intracellular electrical activity were obtained from 161 neurons of the cerebellar cortex of awake cats. In morphologic preparations, two neuronal types were commonly observed, Purkinje and Golgi II cells. In some cases, the dendrites, somata, and axons were filled with HRP, and the neurons could be distinguished readily in light microscopic sections. In other experiments, neurons were incompletely labeled and only dendrites were observed. In addition, more complicated cases arose where intersecting spine-free and spinous dendrites were observed in the same preparation [(11), Fig. 3F]. Thus, it became necessary to examine these preparations with the electron microscope in order to better identify the HRP-injected neurons.

MATERIALS AND METHODS

After intracellular recording from cerebellar neurons of awake cats (11), nanoliter quantities of horseradish peroxidase were pressure-injected into the cells (15, 16). A core of brain tissue containing an injected neuron was obtained 0.1 to 8 h later by applying suction through a 13-gauge needle attached to a syringe (15, 16). The core was then placed into fixative (1.25% glutaraldehyde, 1% paraformaldehyde, and 0.002% CaCl in 0.12 M phosphate buffer, pH 7.3) and was stored overnight at 4°C. After washing 1 h with buffer, 100- to 150- μ m-thick sections of the core were taken on a Sorval TC-2 tissue sectioner. After a brief rinse in buffer, the sections were incubated 20 min in 0.05% diaminobenzidine and 3% H₂O₂. The sections were then dehydrated in a graded series of glycerol before mounting on slides and coverslipping with glycerol. These sections were examined in the light microscope, and photographs were taken of HRP-injected cerebellar neurons before reembedding the sections for electron microscopy. In light microscopic preparations of HRP pressure-injected cerebellar neurons, no alterations of the gross morphology were observed (11). A similar finding has been shown for neurons in the neocortex and other brain regions (3, 4, 7-9, 15, 16, 18, 20, 21).

For electron microscopy, sections containing HRP-injected neurons were removed from slides and placed into buffer. In this study, a total of four partially stained neurons were analyzed as obtained from four separate cores that each received a single HRP injection. Blocks of tissue (1 \times 2

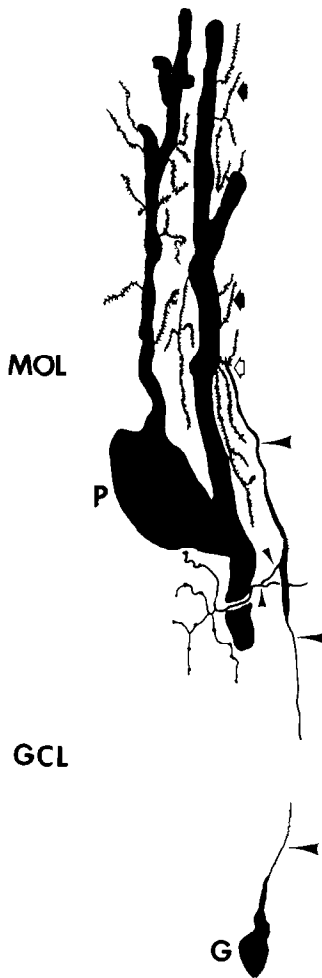
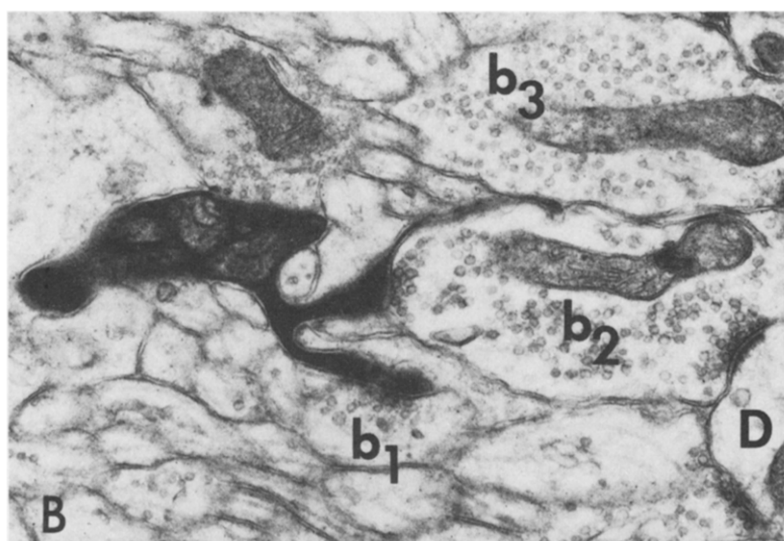
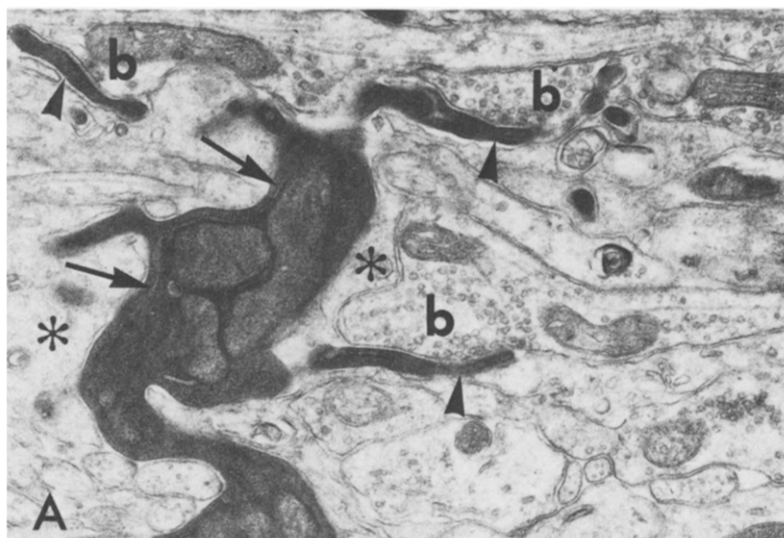


FIG. 1. Drawing of a Golgi II cell and a Purkinje cell from one HRP pressure injection [compare with (11), Fig. 3D]. The Purkinje cell body (P) was located between the molecular (MOL) and granule cell layers (GCL). The entire field of spinous dendrites (solid arrows) from the Purkinje cell is only partially illustrated so that the intersection between dendrites of this cell and of the Golgi II cell may be shown (open arrow). An incomplete staining of the dendrites (large arrowheads) of the Golgi II cell occurred, but an HRP-filled cell body (G) was observed in the GCL. From the HRP-filled dendritic shaft of the Golgi II cell, a fine process which branched at right angles (small arrowheads) was observed. Varicosities occurred along this process which was interpreted to be an axon. $\times 250$.

mm) containing HRP-injected neurons were dissected from core sections and postfixed 1 h with 2% OsO_4 in 0.12 M phosphate buffer. These specimens were stained *en bloc* with aqueous uranyl acetate, dehydrated in ethanols followed by propylene oxide, and then embedded in Araldite.



Semithin 1- μ m sections were taken from the embedded blocks before obtaining ultrathin sections. This procedure allowed for light microscopic observations of HRP-injected somata and dendrites for future orientation of thin sections in the electron microscope. In addition to cutting random thin sections for electron microscopy, serial sections mounted on Formvar- and carbon-coated 1 \times 2-mm slot grids were utilized in order to reconstruct portions of the HRP-containing dendrites. Reconstructions were made by outlining successive profiles of the dendrites on tracing paper.

RESULTS

Purkinje Dendrites. Spinous dendrites containing HRP were commonly observed in the molecular layer of the cerebellum in light microscopic preparations [(11), Fig. 3B]. The arborizations of these dendrites were similar to those for dendrites of Purkinje cells (5, 10, 12) and in some cases the somata of these cells were also filled with HRP (Fig. 1). In electron microscopic preparations of these spinous dendrites, dense amounts of HRP-positive reaction product were observed within dendrites and spines without any spread of reaction product into the extracellular space (Fig. 2). Although the internal structure of HRP-containing Purkinje cell dendrites was partially obscured by reaction product, mitochondria and cisternae of agranular endoplasmic reticulum were still visible. Also, the membranes of these dendrites were well preserved and synaptic junctions were evident. HRP-containing spines from these Purkinje cell dendrites frequently formed synapses with parallel fiber axons but the shafts of these dendrites rarely formed synapses because they were commonly shown to be covered by a layer of neuroglia. These ultrastructural characteristics are similar to those reported in previous electron microscopic studies of Purkinje cells without HRP labeling (5, 10, 12). For purposes of this paper, further

FIG. 2. A and B—electron micrographs of HRP-containing Purkinje cell tertiary dendrites in the molecular layer of the cat cerebellum. A shows a longitudinally sectioned Purkinje cell dendrite filled with HRP. Three adjacent HRP-filled spines (arrowheads) form synapses with parallel fiber boutons (b). Mitochondria and hypolemmal cisternae (arrows) are present within the labeled dendrite, and profiles of astrocytes (asterisks) lie nearby. $\times 26,000$. B shows a cross-sectioned Purkinje cell dendrite filled with HRP reaction product and three profiles of mitochondria. Three boutons (b_1 – b_3) which probably arise from parallel fibers are also present in the field. Two of these boutons (b_1 and b_2) form synaptic junctions with the HRP-filled dendritic spines. One of these boutons (b_2) also forms an asymmetric synapse with a dendrite (D). There is no evidence of extracellular or transneuronal transport of the injected HRP, but the spine associated with bouton b_2 forms an unusual extension between boutons b_2 and b_3 . $\times 18,000$. C and D—electrophysiologic recordings from Purkinje cell shown in B. The recordings in C show cell penetration, spontaneous spiking and a climbing fiber response (arrow). In D, fast sweeps of spike complexes with characteristics similar to those described in (11) are shown.

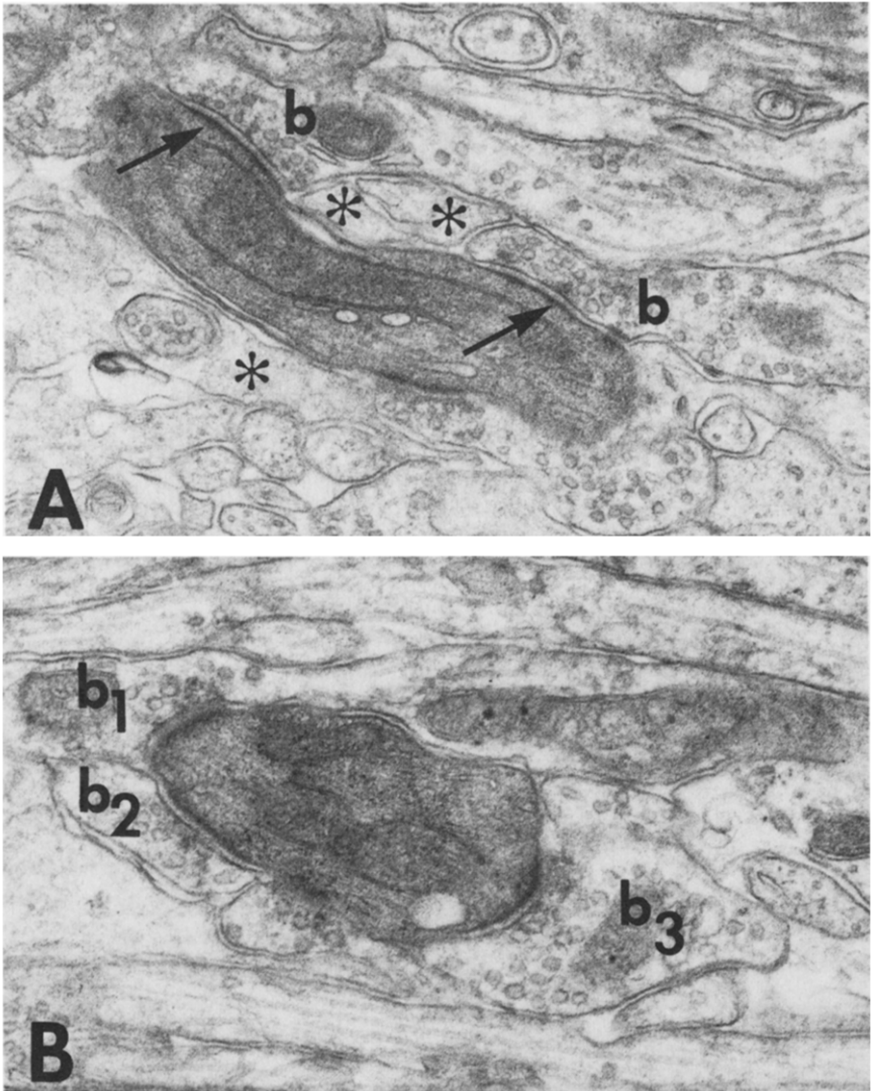


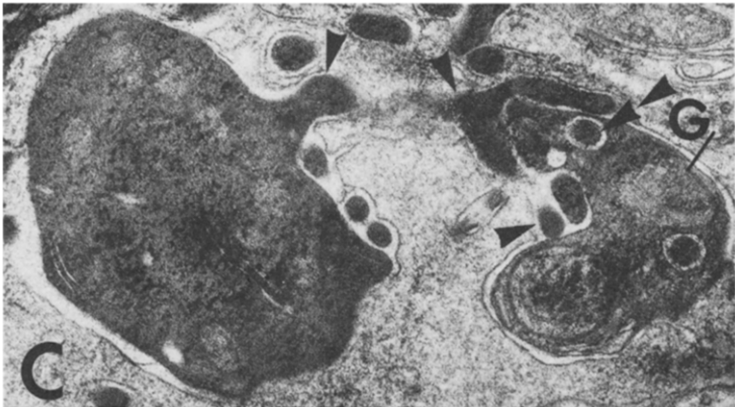
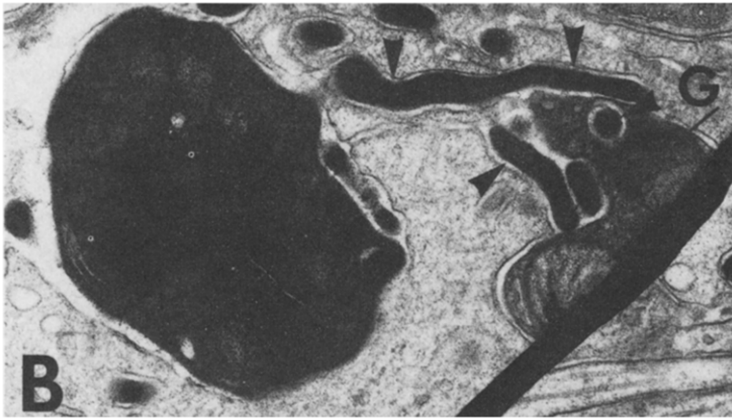
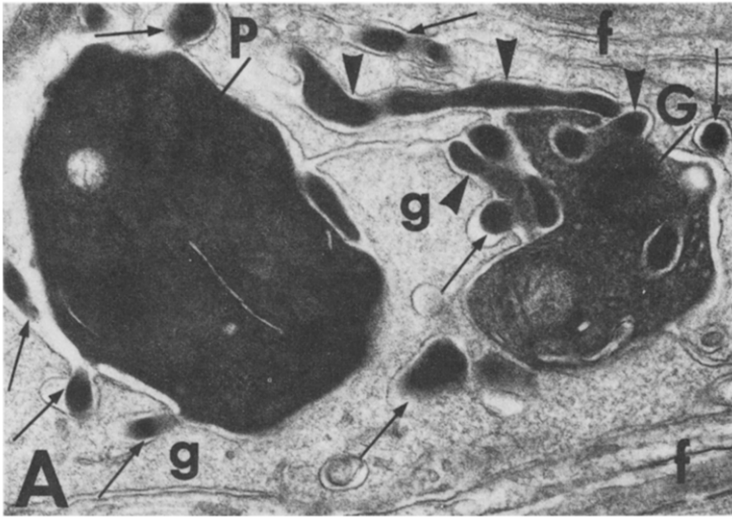
FIG. 3. Electron micrographs of HRP-filled dendritic shafts from Golgi II cells in the molecular layer of the cerebellum. A—a longitudinally sectioned Golgi II cell dendrite. Parallel fiber boutons (b) make synaptic contacts with this dendritic shaft and the paramembranous densities of these synapses are indicated (arrows). In addition to the boutons, glial profiles (asterisks) were also found adjacent to this dendrite. $\times 40,000$. B—a cross-sectioned Golgi II cell dendrite that is surrounded by three synapse-forming boutons (b_1 — b_3). One of these (b_1) is a bouton *en passant* that arises from a parallel fiber. $\times 55,000$.

ultrastructural descriptions will not be given for the somata and axons of HRP-containing Purkinje cells because our purpose was primarily to identify labeled dendrites as part of a physiologic study (11).

Golgi II Dendrites. In addition to the Purkinje cells, another neuronal type was found to contain HRP-positive reaction product. In light microscopic preparations, labeled *aspinous* dendrites in the cerebellar molecular layer were oriented perpendicular to the pial surface and often showed axonal-like processes arising from their shafts [see (11), Fig. 3F]. These axons ramified in the granule cell layer. In some cases either a small or large cell body in the granule cell layer was observed to be continuous with these HRP-containing dendrites (Fig. 1). These characteristics indicated that this neuronal type was a Golgi II cell (5, 10, 12). Electron microscopic observations confirmed this identification in that the HRP-labeled dendrites exhibited characteristics similar to those for dendrites arising from Golgi II cells (Fig. 3). The dendrites were spine-free and had smooth profiles, 1.0 to 1.5 μ m in diameter. They had many synaptic junctions upon their surfaces, and most of the boutons forming these synapses were identified as arising from parallel fibers. HRP-positive reaction product filled the dendrites but it was still possible to observe mitochondria within these profiles. However, unlike the dendrites of Purkinje cells, the Golgi II cell dendrites lacked an abundance of hypolemmal cisternae. Finally, there were a few sites along these dendrites that were covered by neuroglia (Fig. 3A).

Analysis of Intersecting Dendrites. In some light microscopic preparations from experiments where one neuron was recorded and injected, HRP-containing dendrites of Golgi II cells were found to intersect HRP-containing dendrites of Purkinje cells [Fig. 1 and (11), Fig. 3F]. In these cases, cellular identifications were facilitated by the HRP filling of the somata and axons of these neurons. However, in other instances only intersecting spinous and aspinous dendrites were evident in the molecular layer, and dendrites from these cases were analyzed in electron microscopic preparations. Thin sections of the intersecting dendrites (Figs. 4, 5) revealed two different kinds of HRP-containing dendritic profiles that formed a complex of interdigitating processes which were ensheathed by glia. In this dendritic complex, labeled spines resembling those that arise from Purkinje cells appeared to surround the sides of another HRP-containing dendrite (Fig. 4, 5). From reconstructions utilizing a series of serial sections, this latter dendrite was shown to lack spines and a system of hypolemmal cisternae, characteristics of dendrites from Golgi II cells. The other dendrite in this complex was found to be continuous with a structure having all the characteristics of a Purkinje cell dendrite. However, within this dendritic complex Purkinje cell spines did not form synapses with axon terminals.

Using reconstructions obtained from the series of serial sections through this dendritic complex, the putative Golgi II dendrite was shown to be invaginated by finger-like protrusions emanating from these unusual



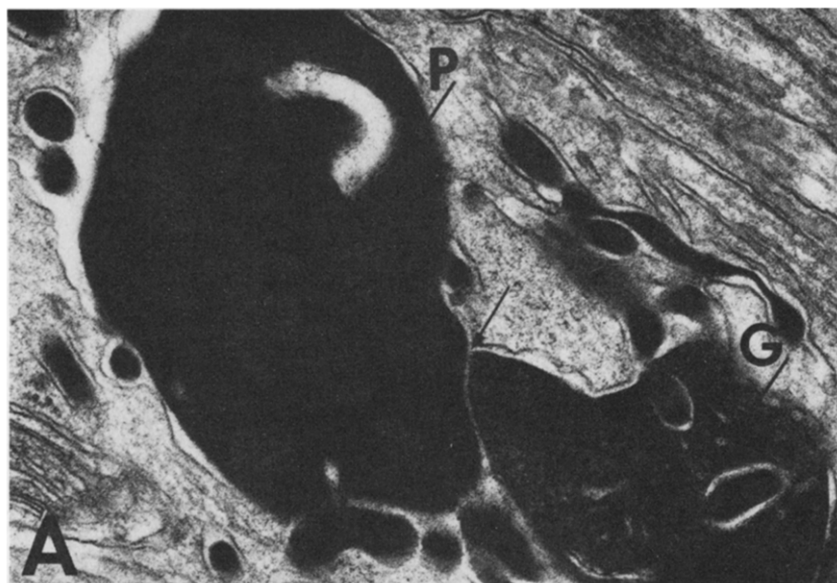
sheet-like, Purkinje cell dendritic spines (Fig. 6). In addition, there were regions of close apposition for these dendrites where two sets of dendritic membranes ran parallel to each other and were separated by a cleft of 20 nm (Fig. 5). In other thin sections from this series the main dendritic shafts were seen to be separated, but the spine-like structures emanating from the Purkinje cell dendrite remained invaginated within the Golgi II cell dendrite, and the entire complex remained ensheathed by glia (Figs. 4, 5). Since the Golgi II cell dendrites did not appear in sections distal to this last site of interdigitation, it is likely that only the terminal ends of these dendrites participated in this glomerular-like structure.

In addition to the above description, this dendritic glomerulus was characterized by other features. For example, the finger-like protrusions that invaginate Golgi II cell dendrites were observed to interdigitate for distances of from 0.25 to 0.8 μm from their sites of penetration (Fig. 6). Some of these finger-like projections were oriented parallel to the long axis of the Golgi II cell dendrite and these two structures were separated only by extracellular space that often contained some electron density. This electron-dense material may represent either HRP reaction product or the characteristic cleft material found at puncta adhaerens. Finally, preliminary results indicated that a 2-nm cleft may exist between the dendritic shafts of the Purkinje and putative Golgi II cells (Fig. 5C).

DISCUSSION

The ultrastructural observations on the HRP-labeled cerebellar neurons studied in this report indicate the importance of electron microscopic preparations for the identification of physiologically recorded neurons. In cases where the somata and axons of HRP-injected neurons are not labeled, an analysis of the dendrites alone must suffice for identification

FIG. 4. Electron micrographs of three consecutive serial sections through a part of the dendritic glomerulus formed by dendrites from Purkinje and Golgi II cells. A—shows the main components of this glomerulus. A *glial sheath* (g) encircles an interdigitating complex of *Purkinje cell spines* (arrows and arrowheads) and *dendrite* (P) and a *Golgi II cell dendrite* (G). Notice that the parallel fibers (f) remain outside this glial-ensheathed glomerulus. A single spine-like structure that is shown in this series to arise from the Purkinje cell dendrite is marked by arrowheads, and can be seen to encapsulate and invaginate the Golgi II dendrite (G). B—shows a part of this same spine (arrowheads) dimpling into the surface of the Golgi II cell dendrite (G). A diagonal fold of the tissue obscures a part of this dendrite in the lower righthand corner. C—shows that this spine-like structure connects with the Purkinje cell dendrite via a thin stalk. Also note that a part of this same spine has invaginated the Golgi II cell dendrite (G) and now appears in a cross section (double arrowheads) surrounded by extracellular space. Another invaginating spine is shown in cross section in the lower right corner of the Golgi II dendrite. All micrographs $\times 36,000$.



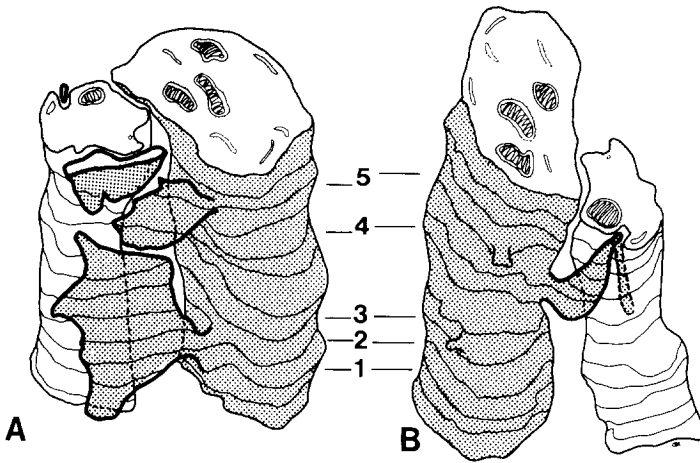


FIG. 6. Reconstruction of the two interdigitating dendrites shown from two viewpoints. A—the view for the reconstruction was taken from the top of each micrograph in Figs. 4 and 5. The Purkinje cell dendrite (stippling) shows parts of three appendages (bold outline). B—the view is the opposite from the view in A. The appendage from this side is shown to have its finger-like protrusion invaginating the Golgi II cell dendrite along its longitudinal axis. Thin sections indicated by the numbers 1 through 5 correspond to those in Figs. 4A, 4B, 4C, 5A, and 5B, respectively. Parts of other appendages were observed in this series of sections but were not illustrated.

purposes. To base this identification strictly upon light microscopic characteristics of dendrites (i.e., branching patterns and the presence of dendritic spines) could be sufficient for identifying neurons in some brain regions. However, for many brain regions these dendritic characteristics would be inadequate for proper neuronal identification, and in these latter instances ultrastructural criteria must be used. In our study, these criteria included the presence of synaptic junctions upon the surfaces of dendritic shafts and/or spines, the identification of the presynaptic component of these synapses, and the presence of intracellular organelles (e.g., mitochondria and hypolemmal cisternae). With these criteria, we identified partially labeled Purkinje and Golgi II cells in the cat cerebellar cortex.

FIG. 5. Electron micrographs from sections 400 nm away from the sections in Fig. 4. A—the site of a 20-nm apposition (arrow) between Purkinje (P) and Golgi II (G) cell dendrites within the dendritic glomerulus. $\times 50,000$. B—the site of dendritic apposition is occluded (box). Note that one of the Purkinje cell spine-like structures (arrow) invaginates the Golgi II cell dendrite (G) very close to this site of apposition. $\times 50,000$. C—an enlargement of the area boxed in B. The membranes of the labeled dendrites are separated by 10 nm (arrowhead) on the left of this figure and approach each other at a point where the separation is only 2 nm (arrow). To the right of this latter point, the membranes are not well defined. A goniometer stage tilted at 30° was utilized in order to resolve this 2-nm site of juxtaposition. $\times 240,000$.

It is interesting to note that many HRP-injected neurons in our study showed labeling only in dendrites. Either the somata of these neurons were not recovered in the cores or the filling of such cells started in the dendrites and the label never spread to the soma and axon. This latter explanation is quite reasonable because in our study action potentials smaller than baseline shifts (undershoots) were frequently encountered (11) and this type of recording has been interpreted to reflect the penetration of dendrites (21). Furthermore, it is unlikely that sections containing somata were not processed because serial sections of all cores were utilized. Therefore, the combination of our morphologic and physiologic data suggests that many of these partially labeled neurons had only their dendrites impaled by the recording micropipette.

In this study we described an unusual spine-like process that arises from Purkinje cell dendrites but differs in its morphology from other spines. For this reason, we suggest that these unusual structures be called dendritic *appendages*. Most spines arise from dendrites as a thin or stout stalk and have at their ends a bulb-like structure that forms synapses with axon terminals (5, 12, 13). The appendages, as shown in reconstructed diagrams (Fig. 6), also arise from dendrites as thin stalks, but they expand to form sheet-like structures that emit one or two finger-like projections. Besides these structural differences, the appendages differ functionally from spines in that appendages are entirely encapsulated by glia and do not form synapses with parallel fibers. In contrast, the appendages appear to lack mitochondria and in this respect, they resemble spines. Based on these observations, a probable function of these appendages may be to place the dendritic shafts of Purkinje and Golgi II cells into close approximation.

Preliminary ultrastructural observations of the dendritic approximation in the glomerulus reveal a separation that is indicative of gap junctions. Similar gap junctions with punctate appositions have been reported previously (2). These junctions were shown to represent sites of electrical coupling and dye transfer (1, 2, 6, 13) and this preliminary observation is consistent with physiologic evidence for electrical coupling between Purkinje and Golgi II cells (11). Gap junctions were also demonstrated in the cat cerebellum between the dendrites and/or somata of basket, stellate, and Golgi II cells (19). It is interesting to note that all four of these cerebellar neuronal types which form gap junctions were shown to contain glutamic acid decarboxylase, the synthesizing enzyme for the neurotransmitter γ -aminobutyric acid (GABA) (14). Since GABA is known to have inhibitory effects in the cerebellum (14), it therefore seems possible that the gap junctions are synchronizing the inhibitory local circuit neurons of the cerebellum. A similar union of inhibitory neurons was suggested for the cerebral cortex (17).

Multiple staining of neurons following iontophoresis of HRP into Purkinje cells was reported previously (9). McCrea *et al.* (9) suggested this phenomenon was due to incidental damage to neighboring neurons by the microelectrode. However, based on the present ultrastructural results that show specific labeling for two neuronal types and a lack of HRP leakage into the extracellular space, the possibility of spread through junctional coupling should be reconsidered. The results of our study demonstrate the usefulness of combining ultrastructural analysis with intracellular electrophysiologic recording.

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