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ULTRASONIC MICRODISSECTION OF RAT CEREBELLUM FOR SCANNING ELECTRON MICROSCOPY

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Abstract

The cerebelli of rats were initially fixed with aldehydes (modified Karnovsky's fixative; 503 mOsM/L) by cardiac perfusion. Blocks of tissue were razor-cut, usually longitudinal to folia, and immersed in the same fluid for 2-4 hours. Three separate methods of treatment followed: (1) immersion in 1% aqueous boric acid, or (2) in 2% phosphate buffered OsO_4 followed by boric acid or (3) in an 8/2 mixture of boric acid and OsO_4. After 18-48 hours immersion the blocks were dehydrated in ascending grades of acetone. They were then exposed to ultrasound in 100% acetone at frequencies of 80 kHz or 40 kHz for 10 to 20 minutes.

Microdissection of cut surfaces (erosion) occurs after all three treatments. It is least extensive after boric acid, moderate after OsO_4 and greatest after the combined mixture. All cerebellar cell types are recognizable as are numerous fibers according to morphology and position. Variable erosion accommodates analysis of different levels of neural organization. In general, structural situations not involving great depth of field are best revealed by H_3BO_3 or OsO_4. Blood vascular relationships to other structures are best demonstrated in deeply eroded specimens.

KEY WORDS: ultrasonication, scanning electron microscopy, central nervous system, cerebellum, neurones, Purkinje cells, granule cells, astrocytes, myelinated fibers, technique

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Introduction

Published work in the scanning electron microscopy (SEM) of the central nervous system (CNS) presently emphasizes its naturally occurring inner and outer surfaces. The inner surface of the brain is expressed largely by the ependymal lining of the cerebral ventricles, with variability centering mainly about the circumventricular organs (Mitchell, 1980; Low, 1982). The external coverings of the brain and spinal cord are most readily studied from the vantage point of the subarachnoid space (Low, 1982). Between these two surfaces lie the gray matter and white matter of classical neuroanatomy: fiber tracts, nuclei and neuropil form an essentially solid mass at gross levels of organization. However, at the cellular level the plasmalemmal contours of glial and neuronal elements constitute a vast and complex domain of hidden surfaces.

A number of techniques designed to facilitate SEM study of these hidden surfaces have been developed (Meller, 1981; evaluated by Scheibel et al., 1981). These have ranged from simple cutting of fresh or fixed tissue to procedures aimed at causing some degree of cellular dissociation without substantial damage to surface contours. Cryogenically induced edema permitted examination of plasmalemmal surfaces normally in close apposition with each other (de Estable-Puig and Estable-Puig, 1975). Conventional aldehyde fixation by cardiac perfusion followed by razor slicing produced interesting results on the cerebellum (Castejon and Carabello, 1980a). This procedure was also varied with cryofixation (Castejon and Carabello, 1980b). Greater depth of exposed surfaces was achieved by "creative tearing" of tissues that were otherwise routinely prepared for SEM (Scheibel et al., 1981). A more recent approach involved microdissection by ultrasound (Arnett and Low, 1984).

Ultrasound used as an aid to SEM examination of animal tissues in general has been shown to be useful, since it reveals surfaces normally covered during the life of the organism (Low, 1981; Highison and Low, 1982). This technique originally depended on hardening tissue by overfixation in osmium tetroxide:

tissues were rendered brittle and susceptible to selective microdissection at 80 kHz (80,000 cycles/sec). An alternate approach, involving cellular dissociation by boric acid (Goodrich, 1942), was adapted for SEM (Vial and Porter, 1975) and further modified by the use of ultrasound for epithelial removal (McClugage and Low, 1982, 1984; Low and McClugage, 1984; Dearing et al., 1984). Selective microdissection of cut surfaces of the CNS was first observed after prolonged fixation in OsO_4 and exposure to ultrasound (Highison and Low, 1982; their Figs. 13, 14). More encouraging results were obtained when osmic acid and boric acid were used separately or in combination (Arnett and Low, 1984).

Materials and Methods

Standard laboratory Sprague-Dawley rats were perfused through the heart with modified Karnovsky's fixative (503 mOsML). The cerebelli were removed and placed in the same fluid for two to four hours. The specimens were then cut transversely or longitudinally with a razor blade and immersed in either 1% aq. boric acid or phosphate-buffered 2% osmic acid for varying lengths of time (16 to 48 hrs). Various combinations of the two solutions, 1% aq. boric acid then osmic acid, or 2% OsO_4 then 1% aq. H_3BO_3 (Low and McClugage, 1984) or mixtures of OsO_4 and H_3BO_3 were also used. After stepwise dehydration in acetone, the specimens were placed in test tubes containing 100% acetone. They were then sonicated at 80 or 40 kHz in tank cleaners for 10 to 20 minutes. Specimens were then critical-point dried using liquid CO_2, mounted on SEM stubs, and sputter-coated with gold-palladium. A Hitachi S-405A and a JEOL JSM-T300 were used for SEM examination.

Results

General Overview

Specimens were prepared to demonstrate the cytoarchitecture of the cortical layers (molecular, Purkinje cell, granular) as well as the central white matter. Although the most consistent and enlightening results were obtained from the boric acid preparations (Fig. 1) interesting findings were also obtained with osmium tetroxide followed by boric acid (Fig. 2). One percent boric acid has been shown to loosen cell junctions, causing cellular dissociation in organized tissues (Goodrich, 1942; Vial and Porter, 1975). This facilitated selective dissection by the sonication process. Cells and their processes were separated from each other, exposing their surfaces for three dimensional viewing. The superficial roughness of the folia in figure 1 is characteristic of specimens treated with boric acid. This type of preparation was extremely useful in demonstrating cerebellar neuronal structure.

The two percent phosphate-buffered osmium tetroxide used by us does not differ substantially from the standard fixative of electron microscopy. Prolonged fixation (24 hrs. or more) has been shown to render the tissues brittle, thus accommodating selective dissection by sonication (Highison and Low, 1982). In the cerebellum all structures were fixed "in situ" by the OsO_4. Subsequent immersion in boric acid further facilitated microdissection. Upon sonication, sizeable pieces of tissue were dissected out, revealing the underlying cytoarchitecture. This was evident from the irregularities and cavitations present on the cut surface of the folia in figure 2. These preparations, although quite different at low SEM magnifications from those treated with boric acid, were quite revealing at higher SEM magnifications. Similar erosion, somewhat less extensive, was produced by specimens fixed only in OsO_4 after aldehyde perfusion.

The basic layers of the cerebellar cortex are illustrated in figure 3. This medium power micrograph shows the molecular layer separated from the granule cell layer by the Purkinje cell layer, all in essentially undisturbed relationships to each other. A small area of central white matter is visible in the lower right hand corner. Purkinje cell bodies and the perikarya of granule cells may be compared for relative size. Some characteristics of each of these layers as they appear in similar preparations are described below.

Molecular Layer

This layer is made up primarily of the dendritic arborizations of Purkinje cells, closely interlocked with Golgi cells and the axons of granule cells (parallel fibers). It is particularly resistant to microdissection. However, in areas where selective tissue removal does occur, some interesting features are revealed. Figure 4 illustrates an outer stellate cell. Microdissection is extensive around the cell body. The surrounding neuropil has been removed by the vibration so as to expose the cell and its processes. Figure 5 is a face-on view of a small cluster of Purkinje cells. A cell body is close to the center of the micrograph and the granule cell layer is to the left. A flattened vein leaves the granular layer, passes in front of the Purkinje cell body and courses through the molecular layer. The orientation of figure 6 is similar to that of figure 5. A Purkinje cell body is at lower left and the greater part of the field is occupied by Purkinje cell dendritic trees, viewed face-on. The main trunk of a Purkinje cell dendrite is shown in detail in figure 7. Two dendritic spines protrude from the main trunk.

Purkinje Cell Layer

This cell layer consists mainly of the flask-shaped perikarya of Purkinje cells and is particularly vulnerable to sonication. Figures 3, 5 and 6 demonstrate both cell bodies and surrounding microdissection. Figure 8 illustrates a group of Purkinje cell bodies from the vantage point of the granule cell layer, here removed by the plane of section. Also visible, in roughly parallel lines, are the dendritic trees of the Purkinje cells representing the basal portion of the molecular layer. The relationship of terminal axonal branches of a basket cell to a Purkinje cell body is shown in figure 9.

Granular Layer

This layer is composed mainly of granule

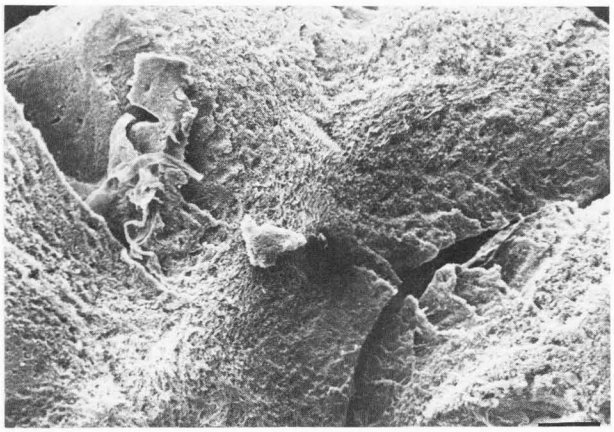

Fig. 1. Cerebellum cut transverse to folia: This boric acid preparation shows only superficial erosion. Compare with figures 2 and 18 (1% aq. H_3BO_3, 20 hrs; 40 kHz, 10 min; Bar = 133 µm).

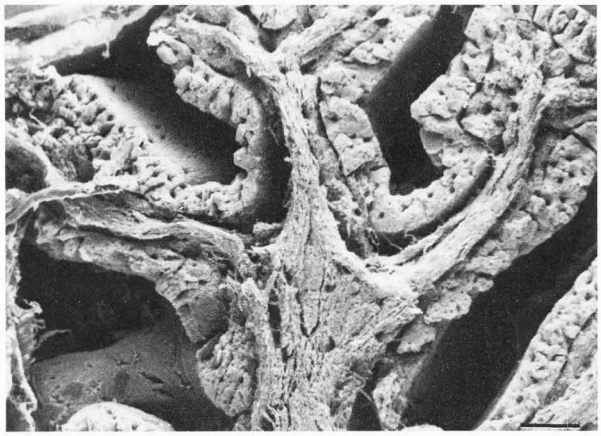

Fig. 2. Cerebellum cut transverse to folia: This osmium tetroxide and boric acid preparation shows moderate erosion. Compare with figures 1 and 18 (2% OsO_4, 16 hrs; H_3BO_3, 24 hrs; 80 kHz, 10 min; Bar = 285 µm).

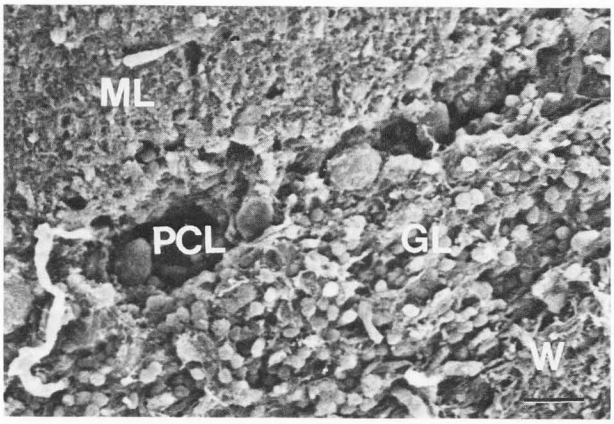

Fig. 3. Layers of cerebellar cortex: The molecular layer (ML) is above. The Purkinje cell layer (PCL) passes from lower left to upper right. The granular layer (GL) is below. A small amount of white matter (W) shows at lower right. (1% aq., H_3BO_3, 16 hrs; 80 kHz, 20 min; Bar = 20 µm).

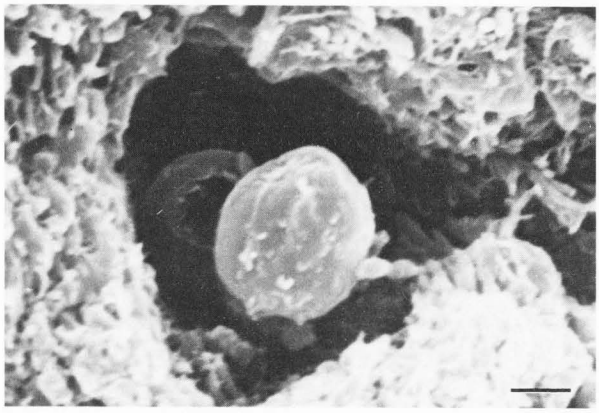

Fig. 4. Outer stellate cell: The nearly spherical cell body is surrounded by extensive erosion caused by sonication (2% OsO_4, 16 hrs; 80 kHz, 10 min; Bar = 1.3 µm).

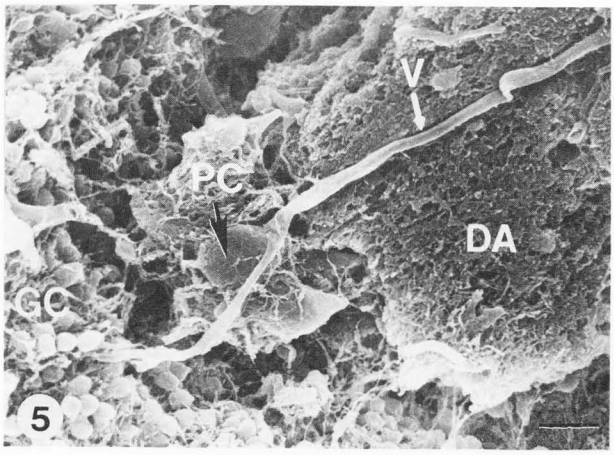

Fig. 5. Purkinje cells (PC): The plane of dendritic arborization (DA) is seen face on with the perikaryon to left of center. Granule cells (GC) are at left. A vein (V) passes from lower left to upper right. (1% aq. H_3BO_3, 19 hrs; 80 kHz, 20 min; Bar = 29 µm).

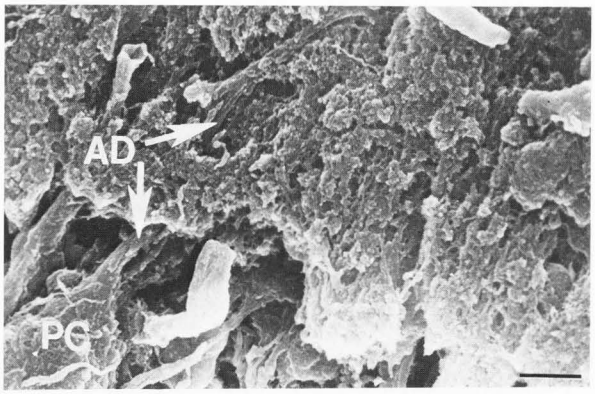

Fig. 6. Purkinje cell dendrites: The cell body (PC) at lower left sends its apical dendrite (AD) upward and to the right. Detail in figure 7. (1% aq. H_3BO_3, 19 hrs; 80 kHz, 20 min; Bar = 10 µm).

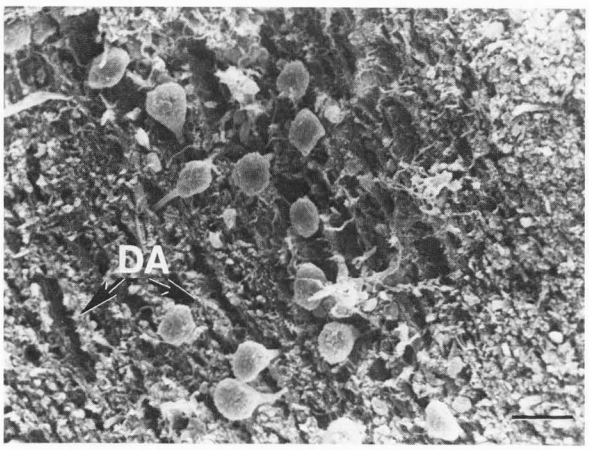

Fig. 8. Purkinje cell bodies viewed from granular layer: Note main trunks of Purkinje cell dendrites and parallel alignment of dendritic arborizations (DA). (1% aq. H_3BO_3, 20 hrs; 80 kHz, 10 min; Bar = 20 µm).

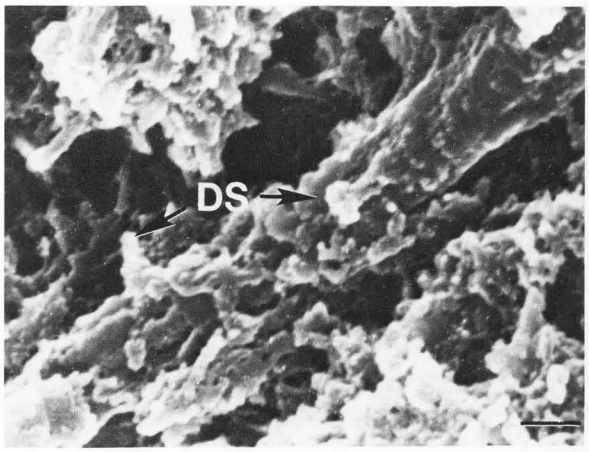

Fig. 7. Dendritic spine, Purkinje cell: This dendrite (detail of figure 6) has been microdissected almost free and reveals two apparent dendritic spines (DS) protruding from the main trunk. (1% aq. H_3BO_3, 19 hrs; 80 kHz, 20 min; Bar = 0.6 µm).

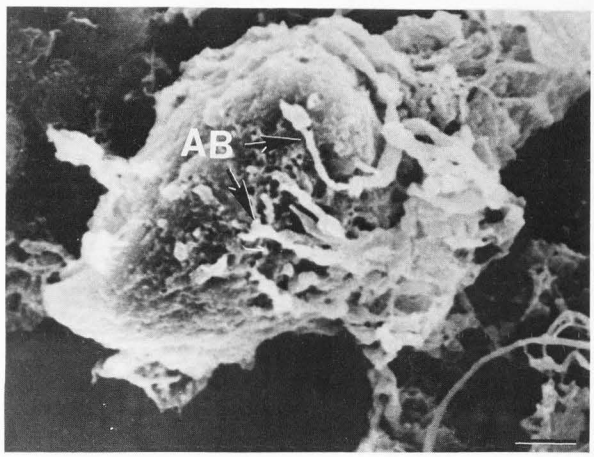

Fig. 9. Purkinje cell with basket cell synapses: These terminal axonal branches (AB) form a "basket" around the Purkinje cell body. (1% aq. H_3BO_3, 19 hrs; 80 kHz, 10 min; Bar = 2.6 µm).

cells, Golgi type II cells, axons of mossy fibers (ending in glomeruli), climbing fibers and Purkinje cell axons. It responds well to microdissection particularly for demonstration of granule cell perikarya and their relationships to terminations of mossy fibers (glomeruli). Figure 10 is an osmic acid preparation showing granule cells as they cluster around the mossy fibers that synapse and pass through the layer. A higher power photomicrograph of a granule cell (Fig. 11) further demonstrates its spherical cell body and shows its thread-like axon as it proceeds into the molecular layer. Occasionally, a basket cell is found below the Purkinje cell layer in the upper third of the granular layer (instead of deep in the molecular layer). The cell indicated on figure 12 illustrates this position of the basket cell as well as its size and shape. The other neurone of the granular layer, the Golgi type II cell, is shown in figure 13. The area around this cell has been removed by sonication and numerous processes can be seen extending into the surrounding tissue.

Figures 14 and 15 demonstrate an important cellular complex of the cerebellum, the mossy glomerulus. Figure 14 shows a cluster of glomeruli with surrounding granular cells. Figure 15 corresponds to transmission electron microscopic reconstruction of the mossy fiber rosette, glial cell, and granule cell relationships. Both micrographs indicate the

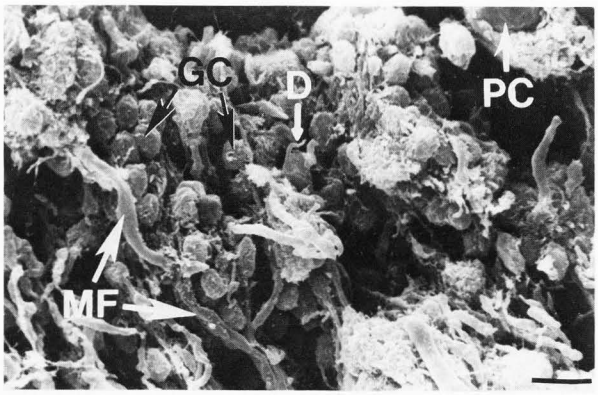

Fig. 10. Granule cells and mossy fibers: The nearly spherical granule cells (GC) cluster about the terminations of mossy fibers (MF). An exposed dendrite (D) is visible. A Purkinje cell body (PC) is at upper right. (2% OsO_4, 16 hrs; 80 kHz, 10 min; Bar = 10 µm).

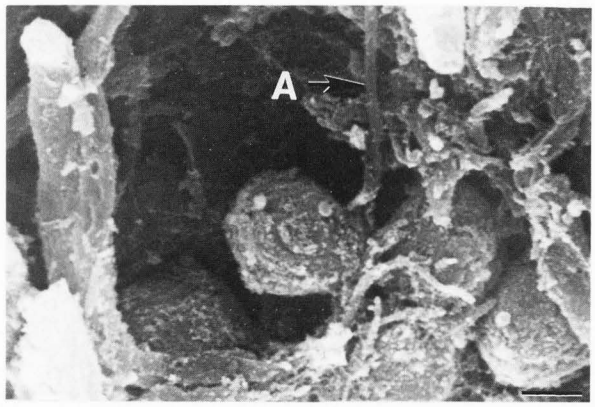

Fig. 11. Granule cell and axon: The vertical course of the axon (A) will bring it to the molecular layer. (2% OsO_4, 20 hrs; 1% H_3BO_3, 24 hrs; 40 kHz, 10 min; Bar = 2 µm).

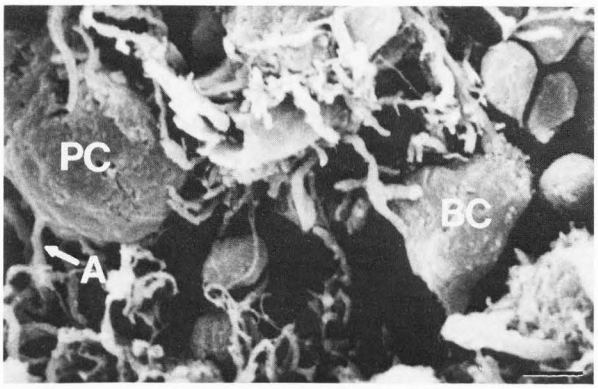

Fig. 12. Basket cell and Purkinje cell: This basket cell (BC) lies in the granular layer just below the layer of Purkinje cell (PC) bodies. A Purkinje cell axon (A) is visible. (1% H_3BO_3, 44 hrs; 80 kHz, 10 min; Bar = 3.3 µm).

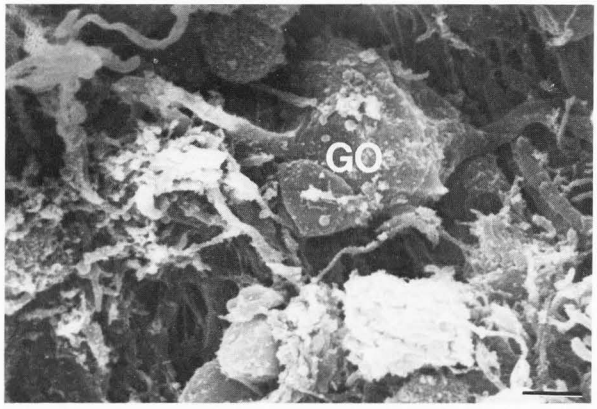

Fig. 13. Golgi type II cell (GO): This cell is recognized here by its size, shape and position. (1% H_3BO_3, 20 hrs; 80 kHz, 10 min; Bar = 2.9 µm).

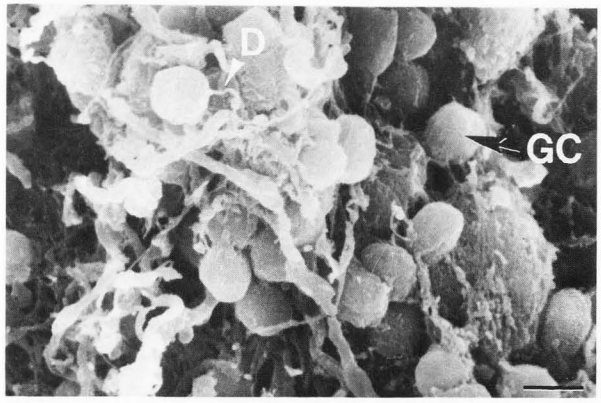

Fig. 14. Mossy glomerulus: Granule cells (GC) cluster around the terminations of mossy fibers. A dendrite (D) is exposed. (1% H_3BO_3, 40 hrs; 80 kHz, 10 min; Bar = 5 µm).

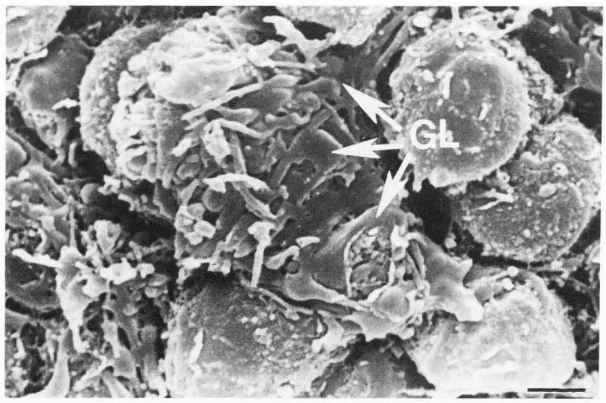

Fig. 15. Mossy fiber termination: Several granule cells frame this complex mossy fiber termination. Glial elements (GL) are plentiful. (2% OsO_4, 16 hrs; 1% H_3BO_3, 24 hrs; 80 kHz, 10 min; Bar = 2 µm).

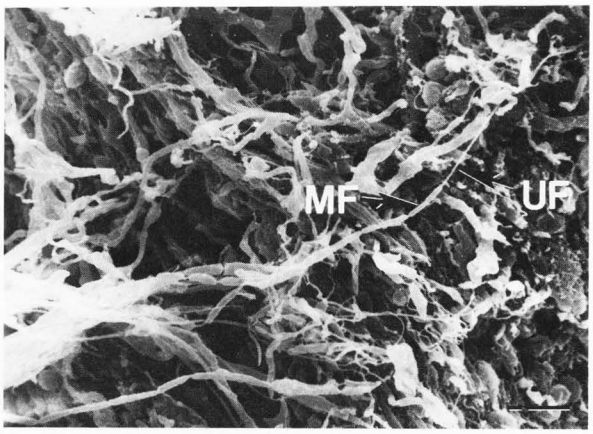

Fig. 16. Myelinated fibers in white matter: One myelinated fiber (MF), coursing from lower left to upper right, becomes unmyelinated (UF) as it leaves the white matter and enters the granular layer. (1% H_3BO_3, 16 hrs; 80 kHz, 20 min; Bar = 10 µm).

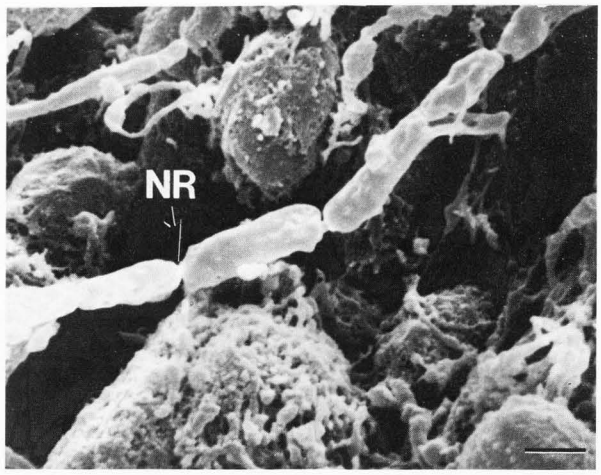

Fig. 17. Myelinated fiber: Nodes of Ranvier (NR) are easily identified on this Purkinje cell axon as it passes through the granular layer. (1% aq. H_3BO_3, 40 hrs; 80 kHz, 10 min; Bar = 2 µm).

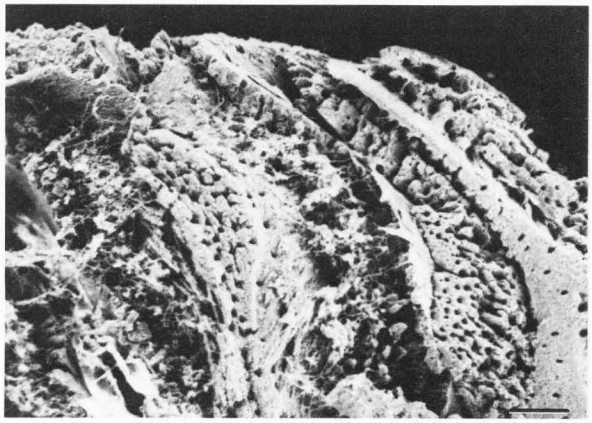

Fig. 18. Cerebellum cut transverse to folia: This preparation from a boric acid and osmium tetroxide mixture shows deep erosion. Compare with figures 1 and 2. (1% aq. H_3BO_3/2% OsO_4-8/2, 19 hrs; 80 kHz, 10 min; Bar = 333µm).

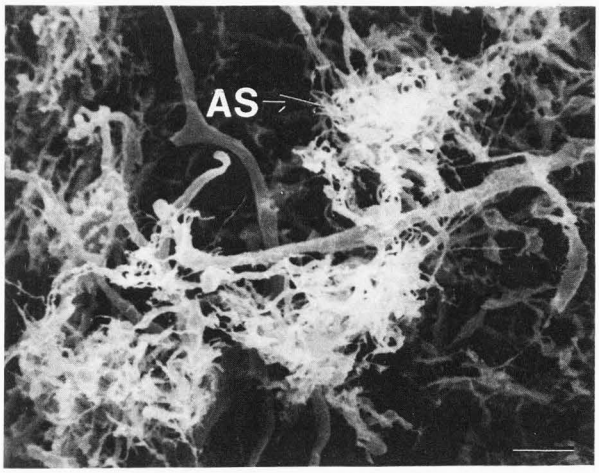

Fig. 19. Astrocytes and blood vessel: The relationship of these astrocytes (AS) to a nearby blood vessel is revealed by deep microdissection. (1% H_3BO_3/2% OsO_4-8/2, 19 hrs; 80 kHz, 10 min; Bar = 13 µm).

possibilities of the technique for demonstration of complex neural relationships.

White Matter

The central white matter of the cerebellum is made up principally of the myelinated axons of afferent mossy and climbing fibers and the efferent axons of Purkinje cells. Figure 16 is a group of fibers showing numerous nodes of Ranvier. One fiber can be seen coursing through the white matter and becoming unmyelinated as it ascends into the granular layer. Figure 17 is a high power view of nodes of Ranvier in a myelinated Purkinje cell axon as it passes through the granular layer.

Neuro-vascular Relationships

Boric acid and osmium tetroxide mixed together in proportions of 8 to 2 respectively yield deeply eroded preparations (Fig. 18). Despite the apparent destruction of the once solid tissue, close examination reveals interesting fields. The relationship of blood vessels to surrounding tissue elements is often revealed. Figure 19 illustrates the relationship of astrocytes to the vasculature.

Discussion

The preparations herein described illustrate the effects of ultrasound on cut surfaces of the cerebellum. Three separate methods of treatment used prior to sonication (boric acid, osmium tetroxide followed by boric acid, and a mixture of the two) are shown to produce distinctly different patterns of microdissection. Each demonstrates in SEM certain known features of cerebellar organization.

These results may be compared with those obtained with other technical approaches, similarly aimed at revealing hidden surfaces of the cerebellum (Castejon and Carabello, 1980a,b; Scheibel et al., 1981). Many similarities of both techniques and results characterize these independent investigations. In general, aldehyde fixation is followed by exposure of deep areas by razor cuts. Despite numerous technical variants among different methods the classic layers of the cortex remain clear. The small, nearly spherical cell bodies of the granular layer show to advantage as do the larger Purkinje cell perikarya. Basket cells and Golgi cells are more difficult to identify with certainty. Among cortical fibers Purkinje cell dendrites and terminations of mossy fibers can be recognized without difficulty. But the remainder of the neuronal fibrous complex poses problems of identification. And the ever present glial cells and their fibers complicate interpretation of at least a few areas in most preparations.

The depth of field present in SEM is particularly valuable when studying the CNS with this instrument (Scheibel et al., 1981). Hence, creative tearing is preferred over simple razor cuts because of greater depth in the torn surfaces. It now appears that microdissection by ultrasonication at 80 kHz offers promise of interesting results for similar reasons. The final contours of sonicated preparations, however, depend largely on procedures operated prior to exposure to ultrasound. Boric acid produces the least erosion of the cut surface, osmium tetroxide a medium amount, osmium tetroxide followed by boric acid somewhat more and an 8 to 2 mixture of the two the greatest erosion. Although localized microdissection of any preparation appears to be haphazard, careful search usually reveals interesting features of neural organization.

The methodology described in this contribution falls far short of technical possibilities that might be put to trial. For example, the classic fixation "hardeners" of early light microscopy are known to yield coherent results on non-neural tissues. Potassium dichromate produces brittleness not unlike that following immersion in osmium tetroxide but subsequent microdissection is less complete (unpublished data). Mercuric chloride hardens samples in such a manner that epithelial layers are peeled off their own basal laminas in undissociated cellular sheets (McClugage and Low, 1984; their Figs. 1,3,4). Interesting theoretic considerations are also offered by pretreatment of living tissues with substrates of known physiological or biochemical activity, this to be followed by the technical procedures herein described.

The sonication technique also offers interesting possibilities for areas of the CNS other than the cerebellum. It has been used with success on the spinal cord and is thought to be well suited to the pituitary, reticular formation, developing brain and spinal cord and other areas. Nor is it an exclusive technique. With modification it could be used concomitantly with techniques like the "creative tearing" of Scheibel et al. (1981). The surface irregularity produced by tearing rather than cutting and the erosion produced by osmium tetroxide and/or boric acid might well be combined to advantage. In summary, with continued refinement, microdissection by ultrasonication offers a multitude of possibilities to investigators interested in the synaptology and the tissue-vasculature relationships of the central nervous system.

Acknowledgements

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Discussion with Reviewers

Rosita F. de Estable-Puig: Which factors in your opinion account for the special vulnerability of the Purkinje layer?
Authors: This is likely due to the presence of many cell somata. Cavitations of various sizes in areas known to be occupied solidly in the undisturbed central nervous system are characteristic of sonicated preparations. We believe that these cavitations start (usually but not always) around a cell body that is larger than the surrounding tangle of neuropil. As sonication progresses the cavitation invades more and more of the surrounding tissue, usually leaving the cell body intact. Further vibration tends to rupture the exposed processes of the cell body and it finally falls free. An empty cavity remains, the cell body being lost to the preparation. This occurs routinely in the Purkinje cell layer.

Rosita F. de Estable-Puig: Was it possible to follow the entire trajectory of the profile identified as an axon in fig. 11?
Authors: No. It soon became obscured by surrounding tissue. Although frequently observed this micrograph illustrates its origin better than others.

Rosita F. de Estable-Puig: Which are the SEM features that allow you to identify the cell in fig. 4 as an outer stellate cell?
Authors: Its position in the molecular layer was the main criterion. Since the surrounding cavitation is large, numerous processes may have been dissected off by the vibration, at or close to the soma.

Rosita F. de Estable-Puig: Have you tried this approach on immersed fixed animal or human cerebellum?
Authors: Animal tissue, yes; human cerebellum, no. The possibilities here depend on two major factors; (1) the slow but long-continuing fixation action of aldehydes and (2) the efficient cellular dissociation caused by boric acid. Early experiments with immersion of fresh cerebelli in boric acid resulted in such extreme dissociation that no tissue block remained. Perfusion with aldehydes (Karnovsky's fixative; 503 mOsM/L) resulted in tissue blocks that responded well to boric acid and subsequent sonication. It had previously been learned that cardiac perfusion with 10% formalin prevented subsequent microdissection, the solution being too strong. This encourages experimentation with freshly autopsied human brains since cellular organization is frequently well retained in such specimens.

M.W. Brightman: Where, in the glomerulus of fig. 15, is the glial component referred to in the text?
Authors: The rough outlines of this structure occupy the greater part of the center of the micrograph. It partly overlaps three granule cells one at upper left, another at lower center and a third in the lower right.

M.W. Brightman: Would not the cell labeled as a Golgi type II neuron (fig. 13) be expected to have many more branches radiating from its cell body?
Authors: Yes. Some may have been broken off by the vibratory insult. But the several processes remaining intact rule out the possibility of its being any other type of neuron in the granular layer.

M.W. Brightman: Is it correct to infer that they (fascicles of parallel fibers) are not preserved by any of the treatments?
Authors: Yes. In our unpublished results with white matter axons are still present within myelin sheaths after OsO_4 but are absent after H_3BO_3. Parallel fibers passing through the dendritic arborizations of Purkinje cell dendrites are represented by small dark holes, often hard to see against the surrounding, rather dark material.

O. J. Castejon: The cell indicated on Figure 12 has been identified as a basket cell. How do you differentiate it from Lugaro cells, which have been described by light microscope in a similar position?
Authors: This cell was identified on the basis of size and position, especially with reference to the nearby Purkinje cell. In SEM, where only surfaces are visible and cell processes often hidden, positive identification of cell types is often difficult.

O. J. Castejon: According to the results, Figures 14 and 15 demonstrate mossy glomerulus. However, the mossy fiber is not indicated. How do you distinguish it from the climbing fiber glomerulus?
Authors: Clusters of granule cells characteristically mark the terminations of mossy fibers, the actual fiber not always being visible. In both figures 14 and 15 the area of termination (or glomerulus) is roughly oval and largely occupies the center of the field. Climbing fibers only pass through and are usually not involved in the formation of a glomerulus. See figures 8-7 and 8-8, p. 170, Core Text of Neuroanatomy, 2nd ed., Williams and Wilkins, Baltimore, 1978.

O. J. Castejon: The characterization of fine nerve processes in the scanning electron microscope is really difficult and offer certain degrees of uncertainties. Similar aspect offers the fine axonal plexuses of Golgi cells and the fine terminal arborization of climbing fibers. On what basis do you identify them as astrocytic end feet?
Authors: The difficulties that you describe do indeed pose problems. Characteristically astrocytes possess many more branches than Golgi cells do dendrites. When viewed "in toto," as in Golgi preparations and in ours, reasonable certainty of identification can be achieved. Our figure 19 should be compared with the Golgi preparations of astrocytes in figures 8-45 and 8-48, p. 320 in Histology by L. Weiss and R. O. Greep, McGraw-Hill, N.Y., 1977.