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Three-Dimensional Morphology of Cerebellar Protoplasmic Islands and Proteoglycan Content of Mossy Fiber Glomerulus: A Scanning and Transmission Electron Microscope Study

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THREE-DIMENSIONAL MORPHOLOGY OF CEREBELLAR PROTOPLASMIC ISLANDS AND PROTEOGLYCAN CONTENT OF MOSSY FIBER GLOMERULUS: A SCANNING AND TRANSMISSION ELECTRON MICROSCOPE STUDY

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Abstract

The present review summarizes the outer and inner surface features of mossy fiber glomeruli in vertebrate cerebellar granular layer as seen by conventional scanning electron microscopy (SEM) and SEM freeze-fracture method. The intracortical trajectory of mossy fibers and their synaptic contacts with granule cell dendrites were traced by the slicing and freeze-fracture techniques revealing the radial distribution of granule cell dendrites around the central mossy rosette. The "en passant" nature of mossy fiber synaptic contacts and the participation of Golgi cell axonal ramifications were demonstrated. The results obtained were compared with available light and transmission electron microscopy data. The freeze-etching technique disclosed the true extension of glomerular neuroglial investment. The proteoglycan content of mossy fiber rosette has been also studied by Alcian Blue staining, enzymatic digestion with testicular hyaluronidase and neuraminidase and Os-DMEDA staining method resulting in the presence of an electron dense material at the mossy fiber axoplasmic matrix and some synaptic vesicles, pre-and postsynaptic densities and cleft substance. The axoplasmic material appears to be constituted by proteoglycans with hyaluronic acid or chondroitin sulphate in their composition. The possible role of proteoglycans in synaptic functions is also discussed. Scanning electron microscopy is a promising methodology for analysis of short intracortical circuits and for the study of complex multisynaptic arrangements.

KEY WORDS: Cerebellar glomerulus, scanning electron microscopy, transmission electron microscopy, synapse, proteoglycan, cerebellum.

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Introduction

The cerebellar protoplasmatic islands and the cerebellar glomeruli were studied at light microscope level by Denissenko (1877), Ramon y Cajal (1889, 1955), Retzius (1892a,b), Lugaro (1894), Held (1897), Bielschowsky and Wolff (1904), Berliner (1905), Estable (1923), Craigie (1926), Jakob (1928), Pensa (1931), Boeke (1942) and Fox and Bertram (1954). Ramón y Cajal (1890, 1926) using Golgi preparations initially described the articulation between the mossy fibers and the granule cell dendrites at the level of the mossy glomeruli and also the participation of the Golgi axonal ramifications. The earlier observations of Ramon y Cajal, later confirmed by the above mentioned authors, led him to postulate the conception of a synapse by gearing. The views of Ramon y Cajal summarized in the Neuronal Doctrine (Ramon y Cajal, 1954) contrasted with the opinion of those supporting the reticularist theory (Golgi, 1874, Pensa, 1931), which conceived the continuity of nerve cell processes forming a diffuse neuronal network. The cerebellar islands were among the first regions of nerve tissue analyzed with the advent of transmission electron microscopy. The pioneering studies of Palay (1956,1961) and Gray (1961) confirmed at the level of the cerebellar glomeruli the validity of the neuronal theory. Subsequent transmission electron microscope studies of the glomerular islands in several vertebrates established the asymmetric or Gray's type I synaptic contacts between the mossy fiber rosettes and the granule and Golgi cell dendritic digits (Fox, 1962; Hamori,1964; Hamori and Szentagothai, 1966; Fox et al, 1967; Sotelo, 1969; Castejón, 1971; Chan-Palay and Palay 1971a,b; Mugnaini, 1972; Palay and Chan-Palay, 1974). According to these studies three-dimensional diagrams of the synaptic connections were made by Eccles, Ito and Szentagothai (1967) and by Mugnaini et al. (1974) in an attempt to provide the three-dimensional image of a multisynaptic complex. In previous papers we have briefly described in human cerebellar cortex using the ethanol-cryofracturing technique (Castejón and Valero, 1980) the synaptic relationship between mossy fibers and granule cell dendrites. In addition in teleost fish cerebellar cortex prepared according to the slicing technique for conventional SEM (Castejón, 1981), we have traced

the course of mossy fibers through the granular layer. In such studies the large depth of focus of SEM allowed us to characterize in a preliminary way the mossy fibers and to compare the results with transmission electron microscopy data. Scheibel et al. (1981) showed the dendritic and axonal structures of the gerbil cerebellar glomeruli using the "creative tearing" method. Arnett and Low (1985) exposed, by ultrasonic microdissection, the granule cells cluster around the termination of mossy fibers and the glial elements involved in the formation of rat mossy glomeruli. More recently we have distinguished mossy fibers from climbing fibers in the cerebellar granular layer (Castejón, 1983, 1986, 1988; Castejón and Castejón, 1988).

Scanning electron microscopy is a promising methodology for studying three-dimensional synaptic relationship. Further investigations in the granular layer neuropil have been carried out in our laboratories using different planes of sections obtained by conventional SEM and the slicing technique and by examining different cleavage planes obtained during the cryofracture process. The aim of the present review is to summarize the outer and inner surface features of a multisynaptic complex: The cerebellar protoplasmic islands and to compare the results obtained with corresponding thin sections and freeze-etching replicas for transmission electron microscopy.

Glycosaminoglycans (GAG), macromolecules earlier considered as characteristic components of the extracellular matrix of tissues have been recognized since 1970 to be also nerve cell constituents (for review see Alvarado and Castejón, 1984). Except for hyaluronic acid, they exist as proteoglycans and have been related to several nerve functions including the storage and release of neurotransmitters (Jones et al, 1982; Salton et al., 1983). In an attempt to demonstrate the localization of GAG at nerve tissue under the electron microscope we had earlier applied, to brain tissue, some polycationic stains as Os-DMEDA and Alcian Blue in combination with osmium tetroxide and some specific enzymatic degradation procedures (Castejón and Castejón, 1972 a,b,c; 1976, 1981, 1988, Castejón et al. 1976). Some of these reports were concerned with the presence, at the mouse cerebellar mossy fiber synaptic endings, of an electron dense axoplasmic material observed also in some synaptic vesicles which was positive to polycationic stains. This mossy material being resistant to neuraminidase and sensitive to testicular hyaluronidase, was suggestive of being either hyaluronic acid or chondroitin sulphate or both. A possible interaction of GAG with some neurotransmitters was also suggested (Castejón and Castejón, 1972a, 1976). Since the present review is dedicated to the three dimensional morphological study of mossy fiber system, we have included these GAG cytochemical results in order to provide more insights into the integral knowledge of this important cerebellar synaptic circuit and also emphasize the increasing functional significance that these macromolecules have recently acquired, especially in cholinergic systems (Jones et al, 1982; Carlson and Kelly, 1983).

Material and Methods

Light microscopy of plastic embedded teleost fish cerebellum (Castejón and Caraballo, 1980a).

The brains of Swiss albino mice and teleost fishes (Arius spixii) were removed and the cerebellar samples were fixed immediately by immersion in 4% glutaraldehyde in 0.1M phosphate buffer solution, pH 7.4 for 4-16h at 4°C; postfixed for 1h in a similar buffered 1% osmium tetroxide solution, dehydrated through graded concentrations of ethanol and embedded in Araldite. Thick sections 1-1.5 µm, obtained with an LKB Pyramitome equipped with a glass knife, were stained with toluidine blue and observed with a Zeiss II Photomicroscope.

Conventional scanning electron microscopy of teleost fish cerebellum. Slicing technique (Castejón and Caraballo, 1980a):

Specimens of Arius spixii weighing 30-82g, kept in aquaria at room temperature were used. Pieces of tissue were fixed: 1) by immersion in 5% glutaraldehyde in 0.1M phosphate buffer, pH 7,4; or 2) by vascular perfusion with 4% glutaraldehyde in 0.1M phosphate buffer solution, pH 7.4; or 3) by immersion with the Karnovsky fixative. Slices of 2-3mm thick were cut with a razor blade and fixed overnight in the same buffered fixative. After washing in buffered saline, the tissue blocks were dehydrated through graded concentrations of ethanol, dried by the critical point method with liquid CO_2 as recommended by Anderson (1951), mounted on copper stubs and coated with carbon and gold-palladium. The specimens were examined in a JEOL 100B Electron Microscope with ASID scanning attachment at 20kV.

Transmission electron microscopy of mouse cerebellar cortex (Castejón, 1984).

For transmission electron microscopy (TEM), slices 1-2mm thick of fish and mouse cerebellar cortex were immediately fixed by immersion in 4% glutaraldehyde in 0.1M phosphate buffer solution, pH 7.4, for 4-16h at 4°C; postfixed for 1h in a similarly buffered 1% osmium tetroxide, dehydrated through graded concentrations of ethanol and embedded in Araldite. Thin sections were stained with uranyl and lead salts and observed with a Siemens Elmiskop I and JEOL 100B electron microscopes.

Freeze-etching and direct replicas of mouse cerebellar cortex (Castejón, 1984).

This technique was applied to the cerebellar cortex of adult Swiss albino mice. The brains were carefully removed and thin 1-2mm slices of the cerebellar cortex were fixed in 1% ice-cold glutaraldehyde 0.1M phosphate buffer, pH 7.2-7.4, for 1h. All cut pieces were immersed in three changes of 25% glycerol in a similar buffer for periods of 1/2h, mounted on gold discs and frozen in Freon at liquid N_2 temperature for 3-5 seconds. They were immediately transferred to a Balzer BAF-301 freeze-fracture unit equipped with an electron beam gun, at -110°C, in a vacuum of 4×10^{-6} or better. Fractured surfaces were shadowed with a layer of carbon-platinum of about 2.5nm thick. Replicas were floated off on water,

cleaned in Chlorox over-night, rinsed in H_2O, bathed in 50% H_2SO_4 and rinsed in multiple changes of water. Cleaned replicas were mounted on grids usually coated with parlodion or Formvar films, and examined with a JEOL 100B electron microscope.

Proteoglycans ultracytochemical study (Castejón and Castejón, 1972 a,b,c):

For this purpose, adult normal albino mice were used. Animals were arranged in three groups so that each group received a different preparation procedure for the electron microscopic study. The first group was processed according to the Benhke and Zelander (1970) staining fixation technique. The cerebellar tissue was initially fixed by vascular perfusion with 2% glutaraldehyde-0.1M sodium cacodylate buffer, pH 6.5 (370 mOsm/l) until the perfusion liquid, flowing from the right auricle, became clear. Perfusion was then continued with a mixture of a similar buffered 2% glutaraldehyde and 0.5% Alcian Blue 8GX (CI No. 74240, Allied Chemical, New York, NY, USA) for approximately 20 min. After perfusion, the brain was allowed to stand in the skull for 1h; then the cerebellum was removed and sectioned into slices, 30-50µm, with a Smith-Farquhar tissue sectioner. Fixation was continued by immersion in a similar glutaraldehyde-Alcian Blue solution for 2h. After rinsing in a similar buffer, the slices were postfixed in 1% osmium tetroxide buffered with 0.1M cacodylate (pH 6.5) for 2h at 4°C. The tissue was then dehydrated through graded ethyl alcohols and embedded in Araldite. Ultrathin sections were stained with both uranyl acetate and lead citrate at high pH and then examined in the electron microscope. Controls consisted of blocks of aldehyde perfused cerebellar tissue, which were postfixed in osmium tetroxide without previous Alcian Blue treatment and processed for electron microscopy as described above. The second group of mice, without previous anesthesia, were beheaded and pieces of cerebellum were fixed by immersion in 4% glutaraldehyde-0.1M phosphate buffer at pH 7.4 for 2h. After initial fixation, the tissues were cut into slices of 30µm thick, washed in 0.1M acetate buffer at pH 5.5 and subsequently immersed for 9-15h at 4°C in a solution of 0.1% Alcian Blue 8GX (ICI, London, England)-acetate buffer at pH 3.5 to which 3% glutaraldehyde had been added. After this step, tissues were processed as previously described.

Some slices of the same glutaraldehyde fixed nerve tissue after washing in phosphate buffer (pH 7.4) and previous to Alcian Blue pH 3.5 staining were incubated in the following enzymes: a) bovine testicular hyaluronidase (Sigma Type V) 0.05% in phosphate buffer (pH 5.5) for 3h at 37°C. b) neuraminidase from Clostridium perfringes (Sigma Type V) 0.05% in phosphate buffer (pH 5.5) for 4h at 37°C. Control experiments were carried out using the respective buffer solution under identical conditions without enzymes.

In the third group of mice, beheaded without previous anesthesia, the brain was quickly removed and the cerebellum sectioned into small pieces, which were fixed in 5% glutaraldehyde-0.05M phosphate buffer (pH 7.4) for 2h at 4°C. Once fixation was completed, the blocks were allowed to rinse for 3h in a similar buffer to which 0.22M sucrose was added. Without secondary fixation the pieces were dehydrated by ethanol and embedded in Araldite. Ultrathin sections mounted on nickel grids were etched by immersion in acetone or dimethylformamide for 20 min and then immersed in filtered 1% Os-DMEDA in aqueous solution for 6-18h at room temperature according to the Seligman et al. (1968) technique. Evaporation was avoided by using covered spot plates. After staining, the ultrathin sections were washed in several changes of distilled water, doubly stained with uranyl acetate and lead citrate and examined in the electron microscope. Poststaining was needed because of the low contrast shown by structures when only the coordination compound was applied. Controls consisted of parallel sections which were not stained by Os-DMEDA but only by uranyl acetate and lead citrate. For morphological controls some samples of aldehyde fixed tissue blocks were also postfixed in osmium tetroxide and prepared for electron microscopy as described above without receiving Os-DMEDA treatment.

Results

The protoplasmic islets appeared in light microscope thick sections of the granular layer as clear areas surrounded by granule and Golgi cells (Fig. 1). The low resolution power of the light microscope does not allow us to discriminate among the structural elements of the granular layer neuropil. Conventional scanning electron microscopy (SEM) of these areas using the slicing technique and gold-palladium coating permitted the identification, at low magnification, of the afferent mossy fibers with their typical rosette formations passing from one granule cell group to another (Fig. 2) and entering into surface contacts with the granule cell dendrites. At higher magnification the large depth of focus of SEM allowed us to trace the parent mossy fibers, to observe their dichotomous pattern of bifurcation and their intricate topographic relationship with the granule cell dendrites (Fig. 3). The mossy fiber rosettes appeared as ovoid or elliptical swellings or expansions on the course of mossy fiber collaterals (Fig. 4). They are clearly distinguished by their high mass density due to the heavy gold-palladium coating and to the absence of neighboring granule cell dendritic twigs eliminated during the slicing procedures. A lateral view of the protoplasmatic islands (Fig. 5) shows the granule cell dendrites converging to the center of the glomerular region. Three to six different granule cell bodies were seen bounding the protoplasmic islands. In this sagittal view of the granular layer the plane of the section disclosed the longitudinal trajectory of the incoming mossy parent fiber entering into a granule cell group and then leaving this region to enter again in a neighboring granule cell group making "en passant" contacts. These "en passant" contacts with granule cell groups were confirmed at higher magnifications (Fig. 6). In addition we observe at the perphery of the glomerular region fine

beaded axonal ramifications in contact with glomerular dendrites. According to their shape, localization and content they have been interpreted as Golgi cell axonal ramifications.

In transverse sections of protoplasmic islands (Fig. 7) up to 12 granule cells were observed surrounding a mossy fiber rosette. Some of these granule cells were observed sending one or two dendrites to the center of the islet and making contact with the mossy fiber rosette. In those sections of the protoplasmatic regions where the plane of the section has disclosed the glomerular region (Fig. 8), the mossy fiber rosette appeared as a varicose expansion surrounded by the terminal dendritic twigs of granule cell dendrites.

Transmission electron microscopy: Thin sections and Freeze-etching direct replicas.

In order to obtain a three-dimensional picture of the cerebellar glomerulus with a higher resolution than that obtained with conventional scanning electron microscopy, the freeze-etching technique for transmission electron microscopy was used. For correlative purposes and proper identification of the mossy glomerular pre-and postsynaptic structures, thin sections of the granular layer neuropil were also studied. Fig. 9 shows two granule cells and a Golgi cell surrounding the mossy glomerular region. As has been widely described by many authors in the last three decades (Palay, 1956; Gray, 1961; Dahl et al., 1962; Hamori, 1964; Fox, et al. 1967, Larramendi, 1968; Uchizono, 1969; Palacios-Pru, 1970, Castejón, 1971; Mugnaini, 1972; Palay and Chan-Palay, 1974) the mossy rosette is the central presynaptic structure of the glomerulus, which appeared surrounded by numerous granule cell postsynaptic dendritic profiles and rather scarce Golgi cell horizontal dendrites. The mossy rosette established asymmetric or Gray's type I synaptic contacts with postsynaptic dendrites. The latter appeared joined by dendro-dendritic attachment plaques. The form of the mossy rosette varied according to the plane of the section, whether being round, ellipsoidal, multilobulate or varicose (Fig. 10) with lateral finger-like projections interdigitated among the neighboring dendritic profiles. Postsynaptic structures appeared lodged in shallow or deep invaginations of the mossy rosette, configurating an articulation or synapse by gearing as classically described in Ramon y Cajal's (1889) pioneering studies.

The mossy rosette contained hundreds of spheroidal synaptic vesicles, clusters of mitochondria, complex or spiked vesicles, dense-cored vesicles, microtubules and neurofilaments.

At the glomerular peripheral region the small Golgi axonal endings could be observed synapsing with granule cell dendrites (Fig.11). The asymmetric synaptic contact exhibited minor dimensions and a small active zone in comparison with the neighboring mossy-granule cell synapses, which allowed us to distinguish both types of synaptic connections. The Golgi axonal endings exhibited scarce spheroidal or ellipsoidal synaptic vesicles. Typical flattened vesicles as those described by Uchizono (1965) in inhibitory synapses were not found.

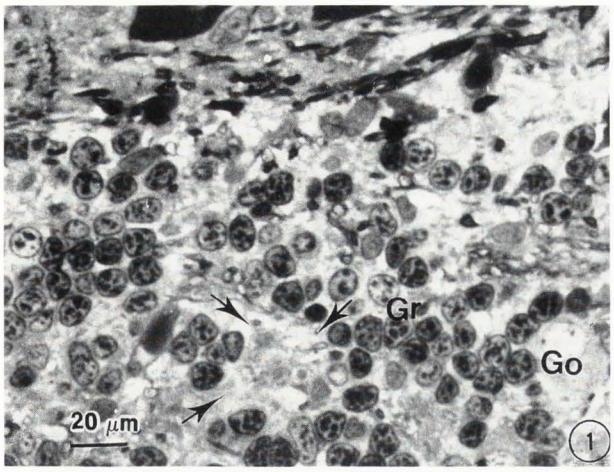
A thin layer of neuroglial cytoplasm appeared to be ensheathing the protoplasmatic islands and currently ubicated in the vicinity of granule cell soma. Gap junctions (Fig. 11) were seen between adjacent neuroglial cytoplasmic processes.

The examination of the freeze-etching replicas provided a three-dimensional relief of the fractured protoplasmatic islands (Fig. 12), in which the central mossy rosette appeared exhibiting a multilobulated shape with the granule cell dendrites partially or totally invaginated. In some areas the cleavage plane exposed the P face mossy rosette plasma membrane exhibiting high density distribution of intramembrane particles (Fig.13). The fractured mossy rosette exhibited as in the thin sections previously described, spheroidal synaptic vesicles, mitochondria and endoplasmic reticulum profiles. The most outstanding contribution of the freeze-etching analysis was the visualization of the true extension of the neuroglial cytoplasm enveloping protoplasmatic islands (Fig. 14), which appeared as a smooth surface cytoplasm extended towards the neighboring granule and Golgi cells. Focal aggregations of intramembrane particles were also observed at the E face dendritic plasma membrane, corresponding to the dendro-dendritic attachment plaques or punta adherentia observed in the thin sections.

Proteoglycan ultracytochemical study.

In the mouse cerebellum fixed by vascular perfusion with glutaraldehyde, stained by vascular perfusion with a mixture of glutaraldehyde-Alcian Blue, postfixed by immersion in osmium tetroxide and sections poststained with uranyl acetate and lead citrate (Fig.15), a fine granular electron dense material was observed deposited throughout the mossy rosette axoplasmic matrix and closely surrounding the synaptic vesicles and dense core vesicle. In addition, some spheroidal synaptic vesicles appeared totally or partially stained. The synaptic complexes and the dendro-dendritic junctions also exhibited an increased electron density. At the level of synaptic complexes continuity between the axoplasmic alcianophilic material and the presynaptic dense projections was found.

A quite similar result was obtained when tissue was fixed and stained with Alcian Blue (pH 3.5) by immersion procedures (Fig. 16), although some tissular alteration produced by sectioning and staining procedure by itself is also seen. Slices of glutaraldehyde cerebellar tissue that had been primarily incubated with testicular hyaluronidase and post-stained by immersion with Alcian Blue (pH 3.5) and osmium tetroxide (Fig. 17) showed its ultrastructure moderately altered giving the impression of a lesser strength of the overall stain. However, the different organelles were identified. An almost complete degradation of the alcianophilic axoplasmic material of mossy fiber rosettes and no dense synaptic vesicles were observed, while the synaptic complexes and dendro-dendritic junctions exhibited a slightly diminished electron density. In the hyaluronidase control tissue, regardless of the notable extractive action of buffer, the extravesicular materi-



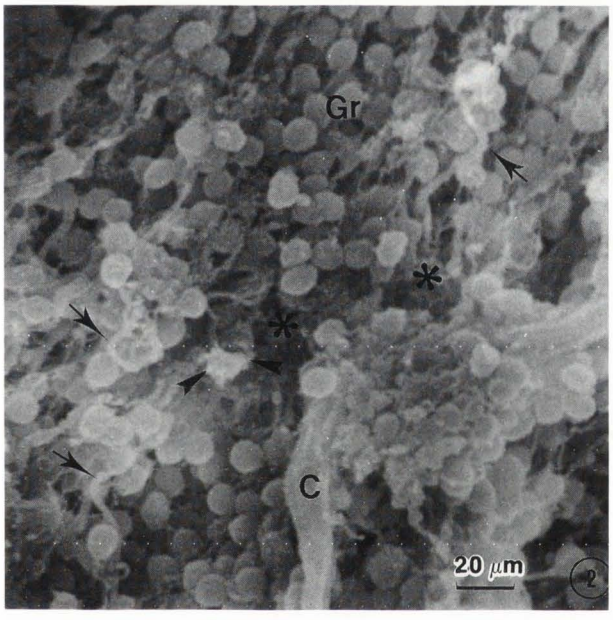
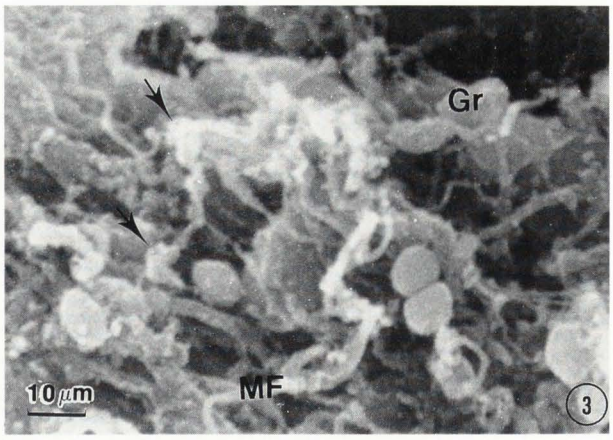


Fig. 1. Photomicrograph of glutaraldehyde-osmium fixed teleost fish cerebellar cortex. Toluidine blue stained-Araldite embedded. Granular layer showing the protoplasmatic islands as clear, free-nuclei areas (arrows) surrounded by granule (Gr) and Golgi (Go) cells. Bar = 20μm.

Fig. 2. Low magnification scanning electron micrograph of teleost fish cerebellar granular layer. Slicing technique. The spheroidal or ovoid granule cells (Gr) appeared surrounding the plasmatic island (asterisks), which show the afferent mossy fibers (arrows). A mossy rosette is clearly distinguished (arrowheads). The outer surface of a capillary (C) is also seen. Bar = 20 μm.



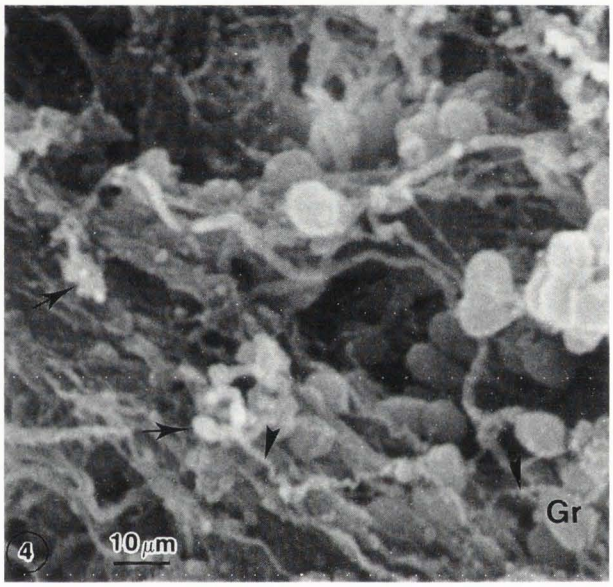


Fig. 3. Low magnification scanning electron micrograph of teleost fish granule cell layer neuropil. Slicing technique. The section of the tissue exhibits the complex organization of the protoplasmatic island. The afferent mossy fiber (MF) shows bifurcations and secondary branches entering into topographic relationship with granule cell (Gr) processes. Some mossy rosettes are also seen (arrows). Bar = 10 μm.

Fig. 4. Low magnification scanning electron micrograph of teleost fish cerebellar protoplasmatic island. Slicing technique. The mossy rosettes (arrows) appears in the first plane as high mass density expansions related to the granule cell (Gr) dendrites (arrowheads). Bar = 10 μm.

al of axoplasmic matrix was still evident (Fig. 18). Neuraminidase treated cerebellar slices poststained with Alcian Blue (pH 3.5) and osmium tetroxide displayed minimal changes in electron opacity when compared with controls. (Figures not shown).

In the tissue fixed only in buffered glutaraldehyde and stained with Os-DMEDA (Fig 19) the limiting membranes of the mossy rosette and those of its organelles were poorly stained. Neverthe-

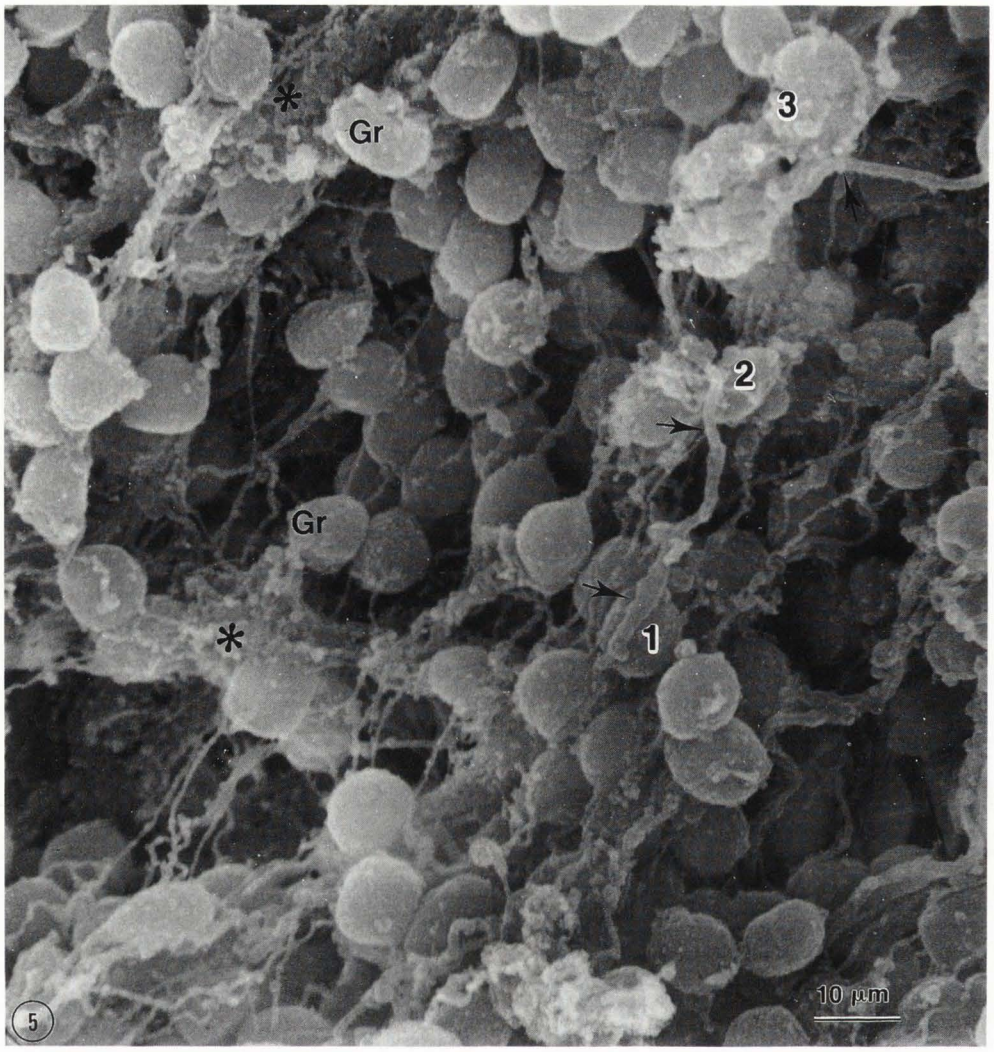


Fig. 5. Low magnification scanning electron micrograph of teleost fish cerebellar granular layer. The outer surface view of two protoplasmatic islands are observed (asterisks) surrounded by several granule cell bodies (Gr). A thick parent mossy fiber (arrows) is observed leaving a granule cell group (1), and entering and leaving successive granule cell groups (2 and 3), suggesting "en passant" contacts with granule cells. Bar = 10 µm.

less a selective electron opacity, high positive reaction, was seen at the mossy rosette axoplasmic matrix which unlike that observed in glutaraldehyde-OsO4 fixed tissue, exhibited a highly contrasted fine fibrillar and granular material. The synaptic vesicles were seen as clear round spaces surrounded by dense axoplasmic material. Increased electron density was also observed at the synaptic active zones, especially cleft material and postsynaptic density and also at the level of dendro-dendritic attachment plaques.

Discussion

Scanning electron microscopy (SEM).

As we have already expressed in previous publications (Castejón and Caraballo, 1980a,b; Castejón and Valero, 1980; Castejón and Castejón, 1981; Castejón 1981, 1983, 1984, 1986; Castejón and Castejón, 1987; Castejón, 1988)the SEM offers the unique possibility of displaying in three-dimensions the remarkable complexity of nerve cell outer and inner surfaces and their interrelationship in situ. In addition conventional SEM techniques, such as the slicing technique, ethanol-cryofracturing technique and the freeze-fracture SEM provide the capacity to scan large areas of brain parenchyma with relatively high resolution and large depth of focus. In the present review the contribution of SEM to study a complex multisynaptic area, such as the cerebellar protoplasmic islets and the cerebellar glomeruli have been shown. Figures 2, 3 and 4 support and extend the earlier descriptions of protoplasmatic islands and cerebellar glomeruli given by Denissenko (1877), Ramon y Cajal (1889),

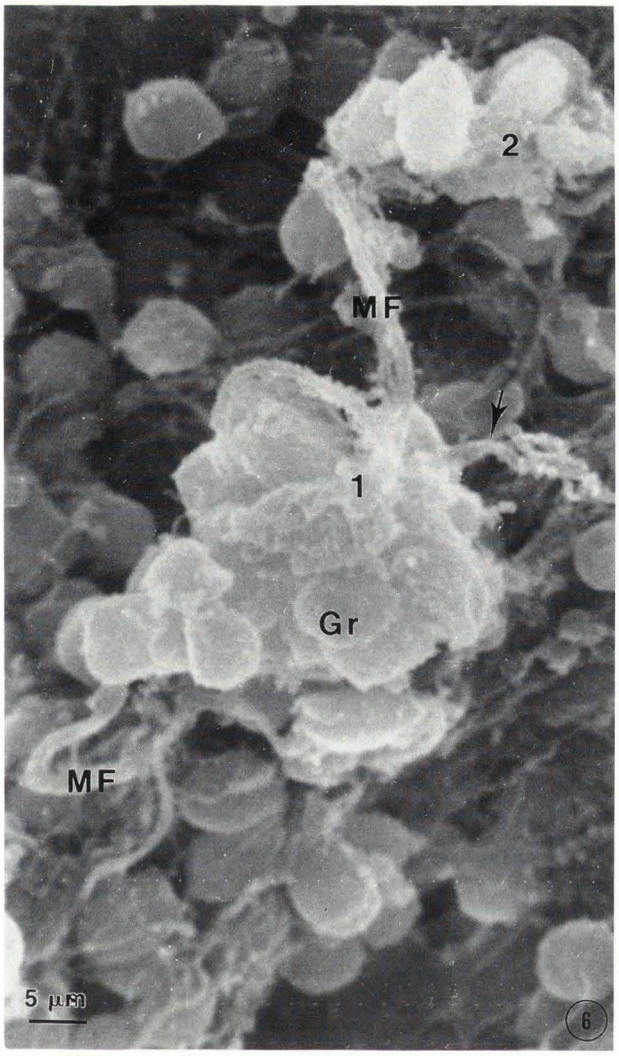


Fig. 6. Higher magnification of two teleost fish granule cell groups (1,2) showing the "en passant" nature of mossy fiber (MF) contacts with granule cells (Gr). In addition a fine contorted cell axonal ramification (arrow) is observed entering the granule cell group 1. This later due to their beaded shape and localization at the periphery of glomerular region has been characterized as a terminal Golgi cell axonal ramification. Bar= 5µm.

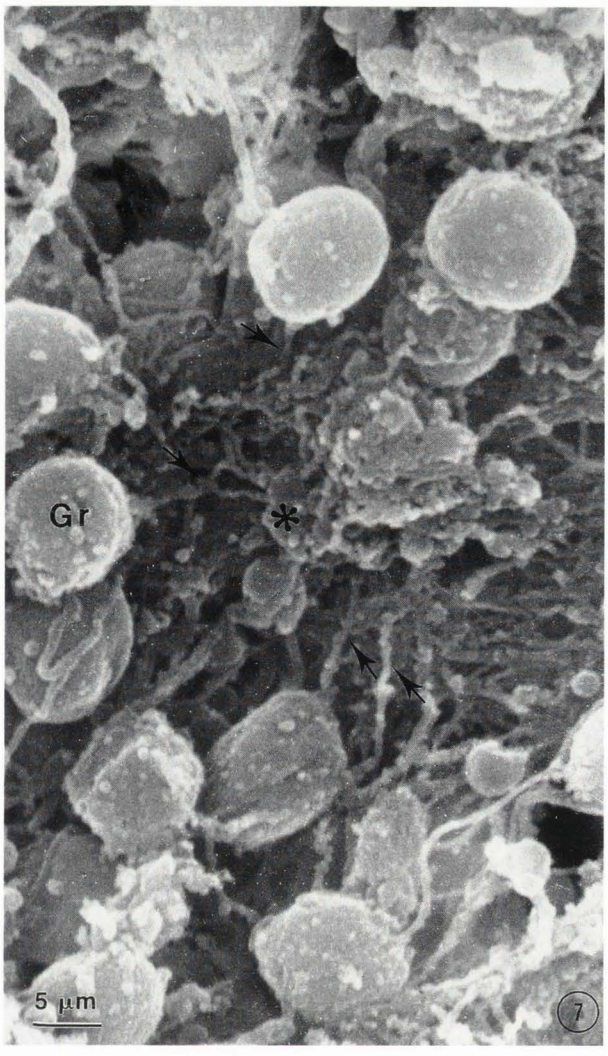


Fig. 7. Transversal section of teleost fish cerebellar glomerular region showing up to 10 granule cells (Gr) surrounding a central mossy fiber rosette (asterisk). The arrows indicate the granule cell dendrites participating in the formation of the glomerulus. Bar = 5 µm.

Kolliker, 1890), Gehuchten (1891), Retzius (1892 a,b), Lugaro (1894), Held (1897), Bielschowsky and Wolff (1904), Berliner (1905), Craigie (1926), Jakob (1928), Pensa (1931) and Boeke (1942).

This study confirms and expands more recent combined Golgi light microscope and transmission electron microscope studies of Fox et al. (1967, 1969), Dahl et al. (1962), Hamori (1964), Szentagothai (1965), Hamori and Szentagothai (1966), Larramendi (1968), Llinas and Hillman (1969), Castejón (1971), Mugnaini (1972), Mugnaini et al. (1974) and Palay and Chan-Palay (1974).

Figures 5 to 8 illustrate a new approach to the neurohistology of cerebellar cortex. The dendritic and axonal fields in a multisynaptic complex are displayed with better definition and sharpness than the Golgi technique at light microscope level. Scanning electron micrographs exhibited a new view of cerebellar granular layer neuropil, which open promising lines of research for qualitative and quantitative studies of the glomerular regions. The first major attempt was to characterize the incoming mossy fibers and to differentiate them from afferent climbing fibers (Castejón, 1988). The mossy rosette expansions and their dichotomous pattern type of bifurcation are the distinctive features of mossy fibers. The climbing fibers do not show enlargements or

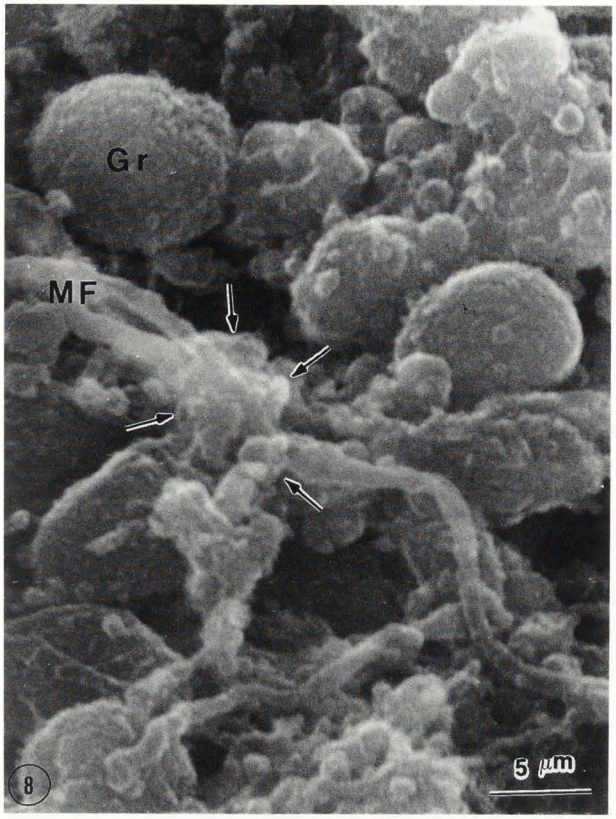


Fig. 8. Teleost fish cerebellar mossy fiber glomerulus. The plane of the section has disclosed the longitudinal course of the mossy fiber (MF). The granule cell dendritic digits appear surrounding the mossy fiber rosette (arrows). Neighbouring granule cells (Gr) are also observed. Bar = 5 µm.

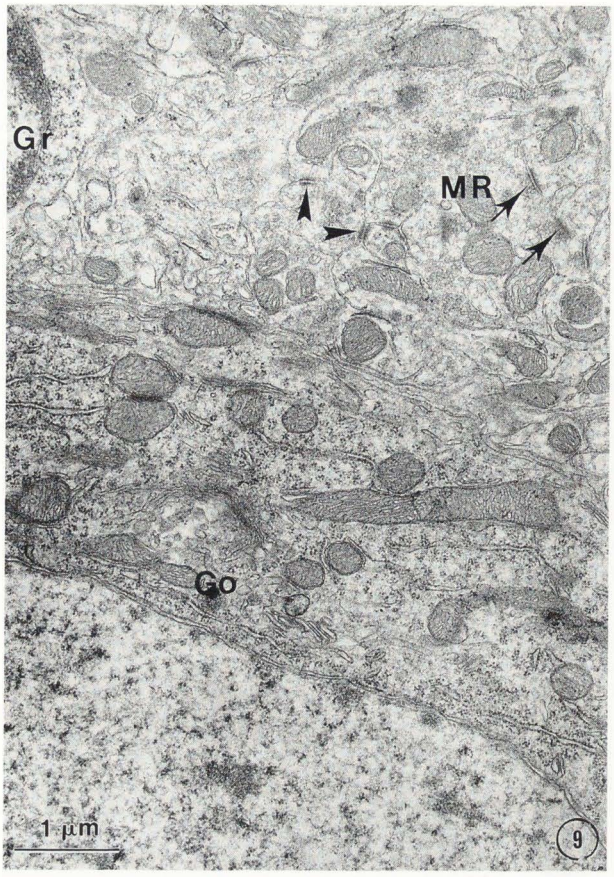


Fig. 9. Transmission electron micrograph of mouse cerebellar cortex. Glutaraldehyde-osmium fixation. A granule cell (Gr) and a Golgi cell (Go) surround a mossy fiber rosette (MR), making axo-dendritic connections (arrows) with granule cell dendritic digits. In addition dendro-dendritic junctions or attachment plaques (arrowheads) are seen between granule cell dendrite postsynaptic structures. Bar = 1 µm.

varicosities, but exhibit a typical cross over bifurcation pattern (Castejón and Castejón, 1987) and continue an ascending course to the molecular layer. The mossy fibers generally remain in the granular layer.

Protoplasmic islets and mossy cerebellar glomeruli.

Figures 5, 6 and 7 illustrate the outer surface views of mossy protoplasmic islands. They show the spatial distribution of 5 to 10 granule cell bodies and their numerous dendrites participating in the formation of the cerebellar glomeruli. However they do not give an idea of the number of glomeruli comprising a protoplasmatic island or if different mossy fiber branches can enter into the same protoplasmic island.

Held (1897) termed cerebellar glomeruli the areas where granule cell dendrites establish connections with rosettes of mossy fibers. Figs. 7 and 8 exhibit transversal and sagittal views of mossy cerebellar glomeruli. They give an idea of the degree of convergence of granule cell dendrites upon a mossy fiber rosette and also of the degree of divergence of nerve impulse at a mossy fiber rosette. We have observed that at a mossy glomerulus up to 18 different granule cells could be counted. Fox et al. (1967) counted up to 15 granule cells entering a single glomerulus. Similarly Eccles et al. (1967) calculated 20 granule cells in synapse with a mossy fiber rosette. These values are in agreement with our observations in slices of teleost fish cerebellum (Castejón and Caraballo, 1980a).

Transmission electron microscopy.

A detailed description of transmission electron microscopy of cerebellar glomeruli was given by Mugnaini (1972) and Palay and Chan-Palay (1974). In the present review thin sections of glutaraldehyde-osmium fixed cerebellar glomeruli have been used as a complementary technique for adequate identification and correct interpretation of scanning micrographs, freeze-etching replicas and as control material for ultracyto-

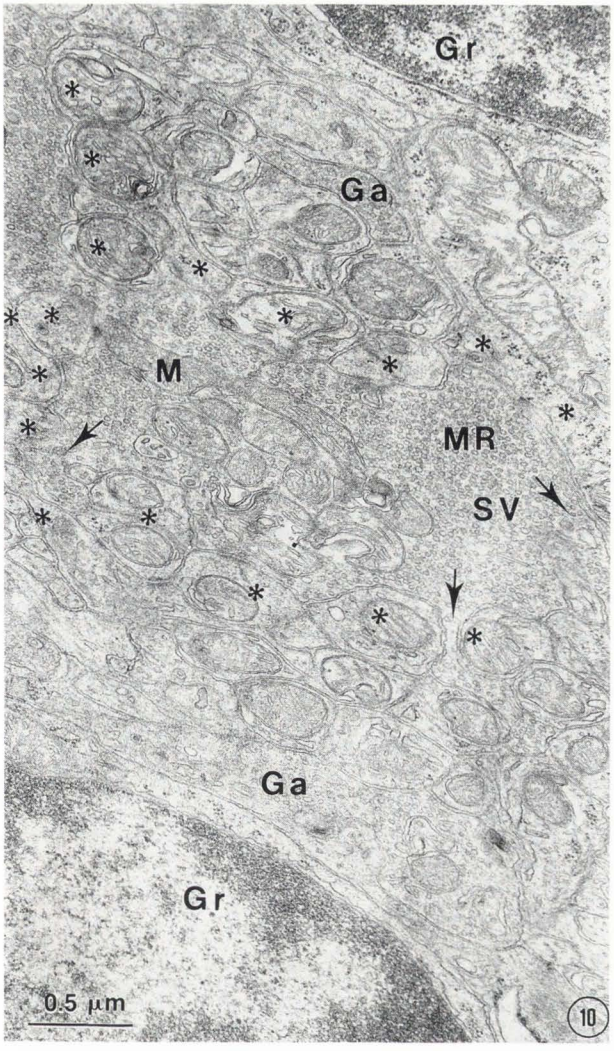


Fig. 10. Transmission electron micrograph of mouse mossy fiber rosette (MR) surrounded by numerous cross sectioned granule cell dendritic profiles (asterisks). Numerous clear, spheroidal or ellipsoidal synaptic vesicles (SV) and mitochondria (M) are observed filling the synaptic varicosities. Note also the collateral finger-like processes of the mossy rosette (arrows) extending toward the periphery of glomerular region. Up to 20 dendritic profiles were counted surrounding the mossy expansion. Another synaptic terminal, apparently a Golgi cell axonal (Ga) ending, containing a mixed population of spheroidal and ellipsoidal synaptic vesicles, is seen between the granule cell dendritic digits and the granule cell (Gr) soma. Bar = 0.5 µm.

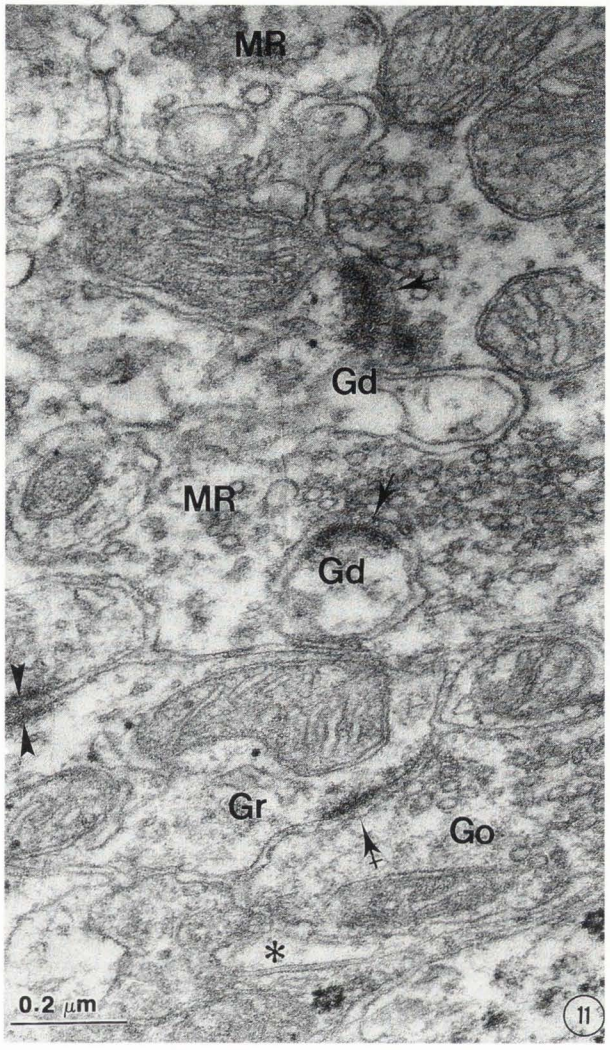


Fig. 11. Higher magnification of a mouse mossy rosette showing the typical Gray's type I axodendritic contact (arrow) between the mossy fiber rosette (MR) and the granule cell dendritic digit (Gd). In addition a Golgi cell (Go)-granule cell (Gr) synapse (cross arrow) is observed at the periphery of glomerular region. The Golgi ending is a small button and its synapse is also of Gray's type I, but the extension of the specialized contact and the dimension of the postsynaptic density is notably smaller than the mossy-granule cell synapse. A dendro-dendritic attachment plaque (arrowhead) is also seen. A gap junction (asterisk) is observed between adjacent neuroglial processes. Bar = 0.2 µm.

chemical study. It is of a paramount importance to emphasize that it was the study of the thin sections of cerebellar islands and the visualization of the synaptic complexes or active sites which gave the validity to the neuronal doctrine of Ramon y Cajal (1955). The thin sections showed that the mossy fiber rosette is the central structure of the glomerulus and that the granule cell dendritic digits invaginate the surface of the mossy rosette making a "synapse by gearing" as formerly described by Ramon y Cajal in Golgi preparations. This type of synaptic connection is clearly visible in freeze-etching replicas, as

Fig. 12. Freeze-etching direct replica of a mouse cerebellar mossy fiber glomerulus displaying the three dimensional relief of the mossy rosette (MR) and the granule cell dendritic digits (Gd). The latter appeared lodged in deep bays of mossy rosette membrane. The real extension of the astrocitary glial cytoplasm (GC) partially ensheathing the glomerulus is appreciated. The fracture plane has disclosed the P face of the mossy rosette membrane (PfMR), displaying the overall distribution of intramembrane particles. Active zones are not distinguished. Bar = 1 µm.

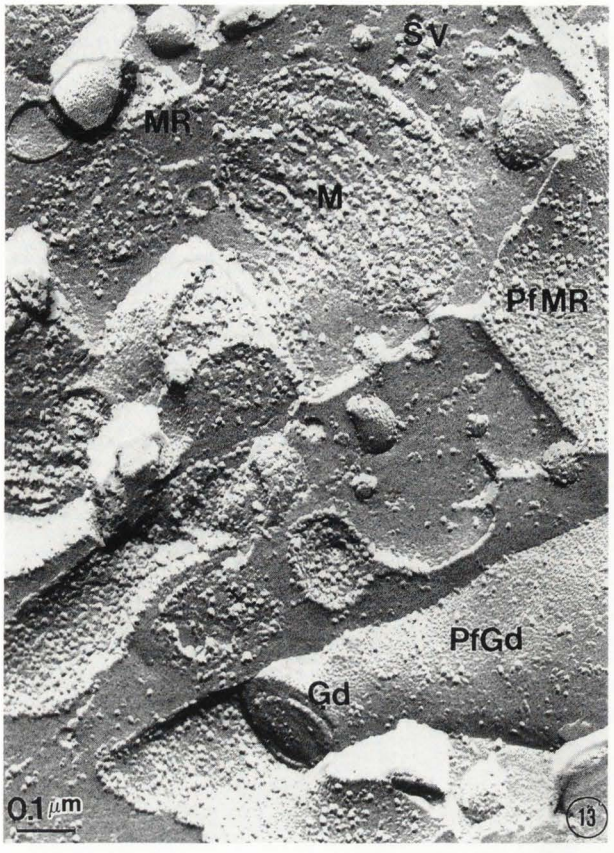


Fig. 13. Higher magnification of a fractured mossy rosette (MR) showing the three-dimensional relief of spheroidal synaptic vesicles (SV) and mitochondria (M). The granule cell dendrite (Gd) appears invaginated within the mossy rosette blocking the visualization of active zones. Intramembrane particles are disclosed in the P face mossy rosette membrane (PfMR) and in the P face granule cell dendritic membrane (PfGd). Bar = 0.1 µm.

illustrated in Fig. 12. The articulation by gearing and the dendro-dendritic attachment plaques give the mechanical support to the multisynaptic complexes. In glutaraldehyde-osmium fixed preparations, the clear spheroidal synaptic vesicles and the mitochondria appeared immersed in a clear axoplasmic matrix. When glutaraldehyde was mixed with Alcian Blue the tinctorial properties of the mossy matrix changed as described in the ultracytochemical study.

Freeze-etching replica offered the true third dimension image of the fractured cerebellar glomerulus. The fractured mossy rosette exhibited a multilobulate contour and the attached granule cell dendritic digits. The cleavage plane exposed the real extension of the glial cytoplasm ensheathing the multisynaptic complex. This feature could not be observed in scanning electron micrographs and was partially seen in thin sections. The neuroglial cells partially encircle the glomeruli suggesting that afferent mossy fibers of adjoining glomeruli may be segregated according to the different kinds of information they bear (Palay and Chan-Palay, 1974). Mossy fiber terminals supplied by primary vestibular afferents fibers have been reported to have a different morphological pattern from other mossy fibers (Brodal and Drablos, 1963), although a later study failed to confirm such a difference (Palay and Chan-Palay, 1974). There are (i) mossy fiber afferents that mediate somesthetics and autonomic signals through the spinal cord and the medulla, (ii) vestibulo-cerebellar mossy fiber afferents that project directly to the cerebellum, and (iii) mossy fiber afferents connected more closely to the cerebral cortex (reticular and pontine nuclei) (Ito, 1984). There is a possible morphological and functional specialization of these different mossy fiber systems as reported earlier by Brodal and Drablos. We have shown in the present study the terminal of the "en passant" portion of the mossy

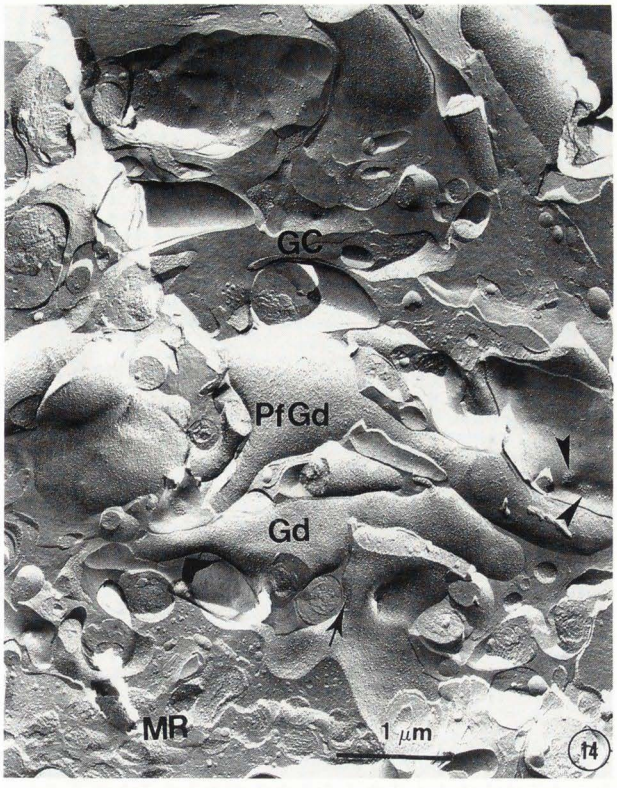


Fig. 14. Freeze-etching direct replica of mouse mossy glomerulus displaying the topographic relationship between a mossy rosette (MR) finger-like process (arrow) and a granule cell dendritic profile (Gd). The P face of granule cell dendritic (PfGd) plasma membrane shows also high density distribution of intramembrane particles. Local aggregation of intramembrane particles (arrowheads) are observed in the E face glial cell membrane. The true extension of the glial cytoplasm (GC) enveloping the glomerular island is noted at the upper part of the figure. Bar = 1 μm.

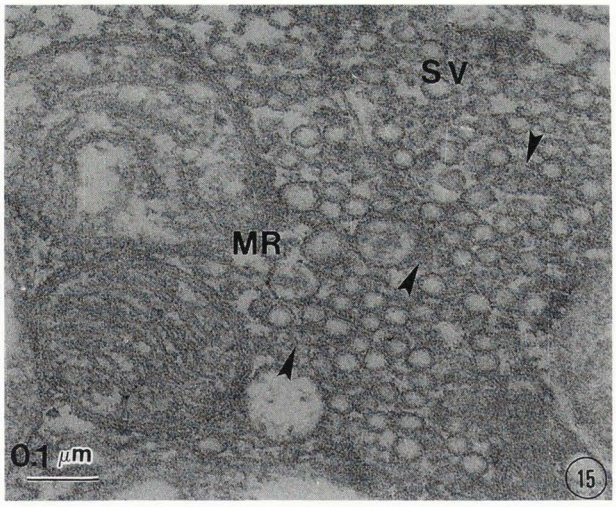


Fig. 15. Transmission electron micrograph of mouse cerebellar cortex primarily fixed by vascular perfusion with a glutaraldehyde-Alcian Blue mixture followed by immersion in a fresh similar solution and post-fixed in osmium tetroxide. Ultrathin section stained with uranyl acetate and lead citrate. The mossy rosette (MR) exhibits clear and dense spheroidal synaptic vesicles. A dense extravesicular material (arrowheads) appears deposited in the matrix of the rosette and surrounding the synaptic vesicles (SV). Some clear vesicles display an inner coat attached to the synaptic vesicle membrane. Bar = 0.1 μm.

fibers of unknown source. Presumably they have different areas of distribution in the granular layer (Eccles et al., 1967). Therefore it is difficult at the present time to correlate mossy fiber three-dimensional morphology as seen by SEM and cerebellar physiology.

Glial cell processes were observed partially ensheathing the glomeruli and separating them from one to another in the protoplasmic island. Our observations could be well correlated with thin section images of lamellar processes of velate protoplasmic astrocytes (Palay and Chan-Palay, 1974).

Proteoglycans ultracytochemical study.

The techniques for the visualization of proteoglycans at nerve tissue are based mainly on the detection of carboxyl and sulphate groups of GAG by polycationic dyes. Intrinsic problems with these techniques are the limitations in their specificity for particular proteoglycans which are partly solved with the application of specific glycosidases, even though these enzyme preparations would be contaminated with other glycosidases or proteases. A more differentiated and specific approach is achieved by the recent use of monoclonal antibodies against well characterized proteoglycan epitopes, as has been reported by Aquino et al. (1984) to demonstrate chondroitin sulphate proteoglycan in nervous tissue. However, whether a monoclonal antibody is specific for a particular type of proteoglycan remains unclear since proteoglycan aggregates have been found to be formed by more than one type of GAG as occurs in rat brain (Ripellino et al. 1989) or in the nerve terminal of the electric organ (Carlson et al. 1986), in which two different kinds of proteoglycan molecules probably sharing the same epitope are recognized by the same monoclonal antibody. We have applied the Alcian Blue and Os-DMEDA stains since they contain 2-4 basic groups which are responsible for their affinity to polyanions. The staining mechanism of Alcian Blue for revealing acidic compounds mainly carboxyl-sulphated polyanions has been long ago reported (for references see Castejón and Castejón 1972a,b, 1988). Also the coordination compound Os-DMEDA has been referred to stain mainly chondroitin sulphate (Seligman et al. 1968; Castejón and Castejón, 1972 a,c, 1976). With both dyes an electron dense material was

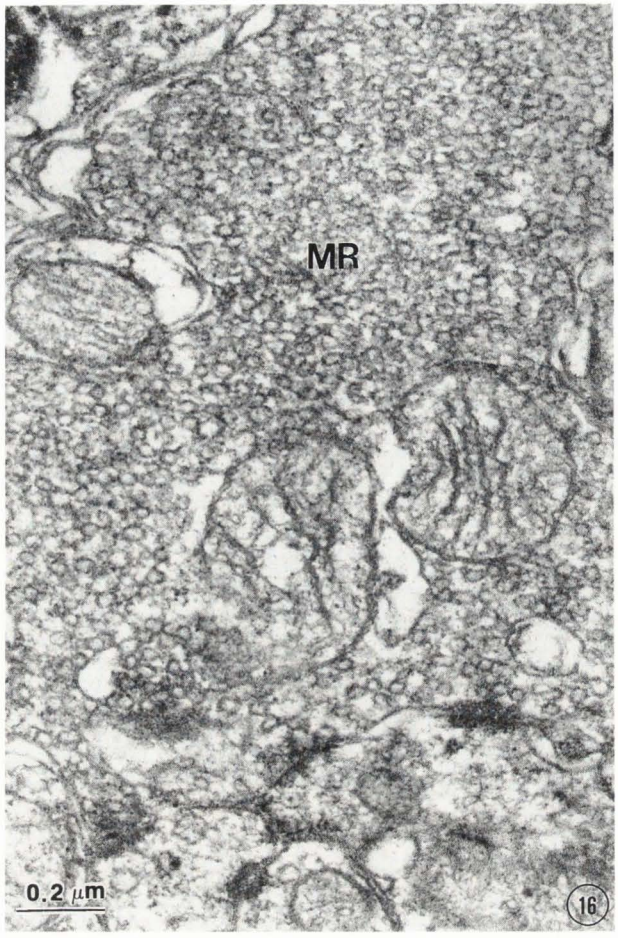


Fig. 16. Transmission electron micrograph of a mouse mossy fiber glomerulus fixed by immersion in glutaraldehyde. Thick sections stained by Alcian Blue-glutaraldehyde and postfixed by osmium tetroxide. Ultrathin sections stained with uranyl and lead salts. The mossy rosette (MR) shows an electron dense homogeneous fine granular material dispersed throughout the axoplasmic matrix and surrounding synaptic vesicles. Bar = 0.2 µm.

observed at the mossy fiber axoplasm, surrounding synaptic vesicles, in the pre- and postsynaptic densities and also at the level of synaptic cleft. The stained substance was presumed to contain both carboxyl and carboxyl sulphated polyanions. The electron density of alcianophilic axoplasmic material as well as that observed in some synaptic vesicles was frankly diminished by the action of testicular hyaluronidase, a glycosidase which splits every second ß (1-4) bond between N-acetylglucosamine and glucuronic acid, being hyaluronic acid and chondroitin 4- and/or 6-sulphate its major natural substrates. Neuraminidase did not affect, to any extent the Alcian Blue reactivity. These findings indicate that carboxyl groups of sialic acids were not the main groups responsible for positive Alcian Blue reaction.

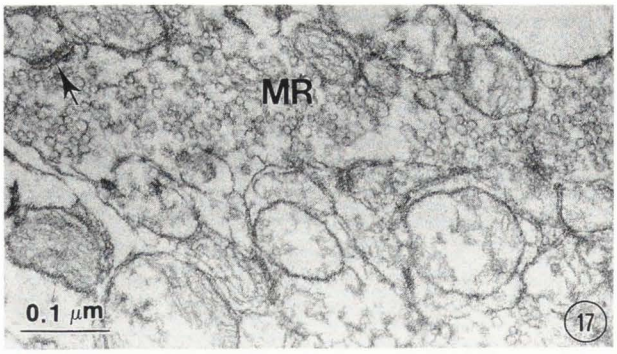
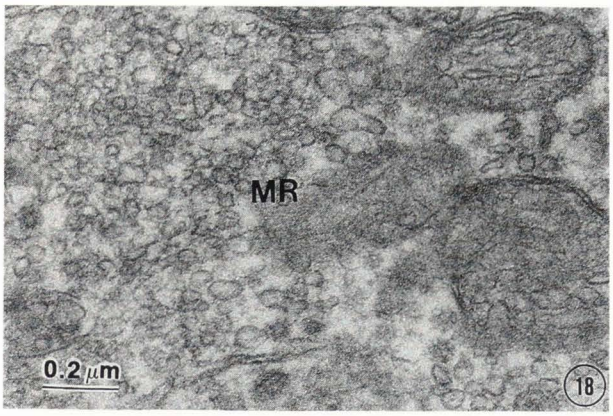


Fig. 17. Transmission electron micrograph of mouse cerebellar cortex fixed by immersion with glutaraldehyde, treated with testicular hyaluronidase, stained with Alcian Blue and postfixed with osmium tetroxide. Despite a lesser strength of the overall staining, the different organelles can be identified. The enzymatic treatment almost completely degraded the electron dense material present in mossy rosette (MR) axoplasmic matrix. The synaptic complex shows also a notably decreased electron density (arrow). Bar = 0.1 µm.

These results are suggestive of the presence, at these sites, of proteoglycans in which either hyaluronic acid and chondroitin sulphates or both are present. It must also be said that the granular or fibrillar material observed at the axoplasmic matrix would not reflect the native state of the proteoglycans but probably a dehydrated, fixation-precipitated form of the macromolecule since most polyanions are highly expanded structures which occupy a large volume in aqueous solution. In the dry state, e.g. in plastic sections after dehydration and embedding, their domains collapse thus bearing little resemblance to the in vivo situation (Scott, 1989). An axoplasmic and pre-synaptic substance with a similar tinctorial characteristic has been recently reported by us in climbing fiber synaptic endings (Castejón and Castejón, 1988). By applying some immunocytochemical techniques to nervous tissue for revealing chondroitin sulphate proteoglycan, Aquino et al. (1984) have shown a positive reaction at the axoplasm of parallel fibers and also in the majority of myelinated fibers at the granular layer of rat cerebellar cortex. Since mossy fibers account for two thirds of the total myelinated fibers in the cerebellar folium (Ito, 1984) it is logical to think that many of the myelinated fibers shown by Aquino et al. (1984) in Fig 4 may correspond to mossy fibers which contain chondroitin sulphate proteoglycans. However, mossy fiber synaptic endings were not shown or studied by these authors. The study of proteoglycans at synaptic endings has been of increasing interest since these compounds seem to play a role in synaptic functions, either by maintaining the electrical stability due to their ability to bind calcium and absorb water, or by limiting the concentration of potassium in the extracellular space by binding it or buffering its action, or controlling the storage and re-



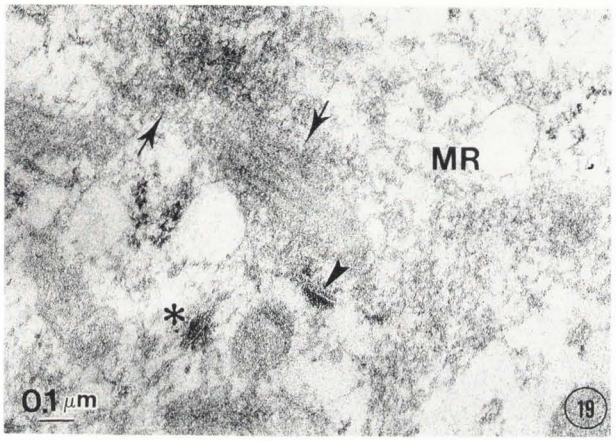


Fig. 18. Control experiment for hyaluronidase and neuraminidase treatment. Incubation of cerebellar slice in the buffer solution without the enzyme. The moderate electron dense material persists in the mossy rosette (MR) axoplasmic matrix being resistant to buffer extraction. Bar = 0.2 μm .

Fig. 19. Transmission electron micrograph of mouse cerebellum etched with dimethylformamide and stained with 1% Os-DMEDA. A granulo-fibrillar material (arrows) is observed in the matrix of mossy rosette (MR). There is a remarkable enhancement of the electron staining of Gray's type I axodendritic contact (arrowheads) and dendro-dendritic attachment plaques (asterisk). Bar = 0.1 μm.

lease of transmitter in synaptic vesicles (for references see Margolis and Margolis, 1977; 1979, Brunngraber, 1979). The apparently selective presence of certain types of proteoglycans in the axoplasm of three cerebellar excitatory systems would rather suggest their involvement in some excitatory mechanisms of neurotransmission. Glutamate and acetylcholine have been reported to be the transmitters released by mossy fibers (Ito, 1984; Garthwaite and Brodbelt, 1989). Recently the presence of some proteoglycans in synaptic vesicles mostly in cholinergic synapses (Stadler and Whittaker, 1978; Jones et al., 1982; Stadler and Dowe, 1982; Carlson and Kelly, 1983; Kuhn et al., 1988) have led to the proposal of several hypothesis as possible explanations of the physiological role of proteoglycans in synaptic vesicles. Jones et al. (1982) have suggested that proteoglycans play a role in vesicle fusion and/or recovery with cholinergic receptors. Carlson and Kelly (1983) relate proteoglycans with the releasing or transport of acetylcholine by binding the neurotransmitter within vesicles. On the other side, Kuhn et al. (1988) have shown certain metabolic stability of proteoglycans within the cholinergic vesicle membrane in synaptosomes of Torpedo marmorata, suggesting that the proteoglycans could rather serve as a stable marker for mature vesicles.

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Discussion with Reviewers

J.E. Rash: There was no data supporting the presence of gap junctions between adjacent neuroglial cytoplasmic processes.
Authors: In Fig. 11, a gap junction can be seen between neuroglial cytoplasm lamellae (asterisk) partially ensheathing the Golgi-granule cell synapse.

J.E. Rash: Fig. 6. How do they know this is a Golgi cell axon?
Authors: Golgi cells in the granular layer exhibit a highly ramified axonal plexus, which can be traced by high optical magnification in Golgi preparations (Palay and Chan-Palay 1974; Castejón, 1976). In an earlier paper, Fox et al. (1967) demonstrated by means of Golgi light and electron microscopy that the Golgi axonal ramifications end in the periphery of the glomerular region. They undercut the cerebellar cortex and allowed the extrinsic afferent mossy fiber to degenerate. The intrinsic, healthy axons remaining in the glomeruli were identified as the Golgi axons. These results were later confirmed by Hamori and Szentagothai (1966) and Mugnaini (1972). With this information in mind we have correlated the fine, beaded axonal ramification with the terminal process of a Golgi cell axonal plexus. We have used the following criteria: localization at the periphery of glomerular region, beaded shape and small size in comparison with mossy and climbing fiber branches.

J.E. Rash: Fig. 9: What is the criteria for dendrites and dendro-dendritic junctions?
Authors: It is well established by Golgi light microscopy since Cajal's studies (1889) and also by electron microscopy (Gray, 1961) that the dendrites of granule cells originate from the cell body and slip between the neighboring granule cells to reach the nearest glomerulus. They could be traced for long distances in electron micrographs of thin sections exhibiting smooth contours, longitudinal microtubules and a terminal expansion termed dendritic claw or dendritic digit. In previous papers (Castejón, 1984, 1988) we have studied by light, transmission and scanning electron microscopy the granule cell dendrites. They exhibit desmosomoid contacts or dendro-dendritic attachment plaques with neighboring dendrites and in some cases appear partially enveloped by neuroglial processes. Therefore, in Fig. 9 we have characterized the small circular or polygonal cross sections surrounding the synaptic rosette as granule cell dendritic digits. They exhibit profiles of endoplasmic reticulum, mitochondria and microtubules and appear as postsynaptic structures which fit neatly into the invaginations of the mossy fiber rosette. Dendro-dendritic attachments plaques appear as symmetrical desmosomoid contacts, which serve as mechanical junctions or adhesion plaques to support the large multisynaptic complex. They were firstly described by Gray (1961) and subsequently confirmed by several workers. Palay and Chan-Palay (1974) describe them as puncta adhaerentia.

J.E. Rash: Fig. 10. How do the authors know that the asterisk identifies dendritic profiles, the "Ga" as a Golgi cell axonal ending, and "Gr" as a granule cell soma?
L.M. García - Segura: The authors should define what are the main criteria for an accurate identification of cell processes in scanning microscopy. For instance, what are the criteria for the identification of Golgi cell axons and granule cell dendrites? What are the main differences between Golgi cell axons and mossy fibers?
Authors: Fig. 10 is a higher magnification of a mossy glomerular region in comparison with that illustrated in Fig. 9. This figure exhibits the classical organization of a cerebellar glomerulus according to all electron microscopic studies already published until now. It is well established (Palay and Chan-Palay, 1974) that the central structure is a mossy rosette expansion crowded with numerous, clear, spheroidal synaptic vesicles. The peripheral collection of circular or elongated profiles lodged over the invaginated surface of the mossy rosette has been identified as the granule cell dendritic digits following the criteria above mentioned. Some of these processes appeared as

postsynaptic structures (crossed arrow) and others exhibit desmosomoid attachment plaques (arrowheads). The Golgi axonic endings have been characterized by their elongated shape, presence of a mixed population of scarce spheroidal and ellipsoidal synaptic vesicles and localization at the periphery of the glomerulus, between the granule cell dendritic digits and the granule cell soma. The granule cell soma exhibits the typical features of the small microneurons: a scarce ring of perinuclear cytoplasm and compact heterochromatin masses within the nucleus. Compare with Fig 9, which displays the organization of a macroneuron, the Golgi cell, with abundant cytoplasm, well developed rough endoplasmic reticulum, numerous mitochondria and a vesicular nucleus. The main differences between Golgi cell axons and mossy fiber will be described in the next answer.

J. E. Rash: Fig. 11: How can they distinguish between a Golgi cell (Go) - granule cell synapse and a mossy fiber (MR)-granule cell synapse? To this reviewer the Go presynaptic process looks no different than the "MR" labeled structure.
Authors: Mossy fibers are large, extrinsic afferent fibers which form large terminal or "en passant" presynaptic endings, about 10-20 microns long, that correspond to the rosette and also exhibit hundreds of spheroidal synaptic vesicles. The rosettes appeared as round or fusiform enlargements with finger-like projections, or as a central expansion with filiform appendages or as elongated cones or tubes with filamentous processes. The mossy rosettes form the central core of the glomeruli. On the contrary, Golgi cell axons are intrinsic fibers of the granular layer, which divide and ramify profusely in the granular layer forming an axonal plexus. This complex fine arborization has a characteristic beaded shape in Golgi light microscope preparations and in transmission electron microscope thin sections. This fine arborization ends at the periphery of the glomerular region by means of small, terminal buttons in contact with granule cell dendrites. The Golgi axonal endings are about 1-4 microns in diameter, spheroidal or elliptical in shape, with scarce número of spheroidal and flattened synaptic vesicles. These axodendritic contacts are always ubicated between granule cell dendrites and granule cell somata, at the periphery of the glomerular region. In relation to your question it is relatively easy to discriminate between both types of synaptic contacts. Mossy fiber-granule cell synapse is a large multisynaptic contact of the glomerular type. Golgi cell-granule cell synapse is a small, monosynaptic axo-dendritic contact. The mossy fiber-granule cell synapse is asymmetric or Gray's type I with invaginated, large synaptic complexes, a widened synaptic cleft and a thick plaque of filamentous material attached to the postsynaptic dendritic surface. The presynaptic membrane exhibits a small cluster of round synaptic vesicles (S type) and presynaptic dense projections. The Golgi cell-granule cell synapse is generally the flat type with a small synaptic complex, symmetric or asymmetrical (Gray's type I or II), 0.1 - 0.3 µm in diameter, without widening of the interstitial cleft. From the physiological point of view mossy fiber-granule cell synapses are excitatory junctions and Golgi cell-granule cell synapses are inhibitory in nature (Eccles, Ito and Szentagothai, 1967).

J. E. Rash: Fig. 12. How can they distinguish dendritic digits as granule cell rather than Golgi cell neurites? The glial cell encapsulation is not clear and convincing.
Authors: Golgi cell exhibits ascending and horizontal dendrites (Castejón, 1976). Ascending dendrites run vertically toward the molecular layer and horizontal dendrites remain in the granular layer to establish synaptic contacts with mossy or climbing fibers (Mugnaini, 1972). In thin sections we cannot distinguish Golgi and granule cell dendritic digits as postsynaptic structures at the level of the mossy glomeruli. However the dendritic trunk of the Golgi cell give off relatively few horizontal branches in the granular layer. Therefore most postsynaptic dendritic digits correspond to granule cells. As depicted in Fig. 7, scanning electron microscopy clearly evidentiates this data and support most Golgi light microscopic studies. Therefore, in general we assume that most postsynaptic dendritic digits belong to granule cell dendrites. In this context the ratio Golgi cell granule cell in granular layer should be considered. Palkovitz et al. (Palkovitz, M.; Magyar, P. and Szentagothai, I, Brain Res 32: 15-30, 1971b) counted 1 Golgi cell for every 5701 granule cells, which gives an indirect idea of the scarce Golgi cell contribution to the formation of mossy glomeruli.

The mossy glomeruli appear partially encircled by neuroglial cell cytoplasm. This data has been reported in most transmission electron microscopic studies of mossy glomeruli. Palay and Chan-Palay (1974) express that the protoplasmic astrocytes subdivides the granular layer into compartments. Fig. 12 is a freeze-etching replica in which the cleavage plane has disclosed only the glial processes covering one third of the lower glomerular surface. On the contrary in Fig. 14, the cleavage plane showed the glial ensheathing of the upper glomerular surface. Consequently, figures 12 and 14 show the partial glial encapsulation of the glomerular region.

L. M. Garcia-Segura: What are, in the opinion of the authors, the main limitations of scanning microscopy for neurocytological studies?
Authors: There are several limitations for the use of scanning electron microscopy, in the study of nerve tissue: a) Sample processing: Conventional scanning electron microscopy introduced a wide range of artifactual damage induced by glutaraldehyde fixation, such as blister formation. Critical point drying produces a 10-20% retraction due to the high aqueous content of nerve tissue (up to 60%). This shrinkage modifies the outer and inner neuronal surface. The metallic coating, for example thick gold palladium, obscures outer surface details. In addition the charging effect during specimen observation is a serious concern. The use of cryofracture technique allowed us to expose some hidden surfaces of neurons and neuroglial cells, but these are

the results of fortituos cleavage planes. The advent of high resolution scanning electron microscopy offers new and promising methodologies, such as delicate specimen preparation, use of new filaments (field emission emitters) and different instrumental parameters, which give the possibilities for observations in a range similar to that obtained with transmission electron microscopy at high magnification. Scanning electron microscpy is just a method for three-dimensional observation of nerve tissue. But proper identification of nerve cell types and tracing of intracortical circuit require the use of correlative techniques such as Golgi light microscopy, transmission electron microscopy and freeze-etching technique (Castejon, 1988).