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SCANNING ELECTRON MICROSCOPE, FREEZE ETCHING AND GLYCOSAMINOGLYCAN CYTOCHEMICAL STUDIES OF THE CEREBELLAR CLIMBING FIBER SYSTEM

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Abstract

Mouse and teleost fish cerebelli were processed by the freeze-fracture methods for scanning and transmission electron microscopy in order to study the three-dimensional morphology and intramembrane features of climbing fiber-Purkinje spine synapses. In addition, Alcian Blue and ruthenium chloride stainings were applied to mouse cerebellar tissue to investigate the polyanion composition of these excitatory synapses under the transmission electron microscope. In the granular layer, tendril and glomerular collaterals of climbing fibers were observed. In the molecular layer climbing fibers exhibited a characteristic crossing-over or arborescence pattern type of bifurcation, Scheibel's collaterals and multiple thorn synapses with Purkinje spiny dendrites. At the synaptic active zones of climbing fiber-Purkinje spine synapses the freeze-etching replicas showed focal aggregates of intramembrane particles at the E and P faces of the pre- and post-synaptic membranes. Membrane protuberances and pits were also observed at the pre-synaptic membrane. Ultracytochemical study of the climbing fiber synaptic varicosities revealed an Alcian Blue and ruthenium chloride positive material which appeared at the axoplasm surrounding the synaptic vesicles, at the pre- and post-synaptic densities and in the synaptic cleft. The axoplasmic material was sensitive to testicular hyaluronidase, therefore it would correspond to glycosaminoglycans (hyaluronic acid and/or chondroitin sulphates), which have been earlier reported in other cerebellar excitatory systems as those of mossy fiber-granule cell and parallel fiber-Purkinje dendritic spine.

KEY WORDS: Cerebellum, Climbing fiber, Excitatory synapses, Scanning electron microscopy, Freeze-etching, Glycosaminoglycans cytochemistry.

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Introduction

Light and transmission electron microscopic studies of vertebrate cerebellar cortex (Carrea et al., 1947; Scheibel and Scheibel, 1954; Szentagothai and Rajkovits, 1959; Larramendi and Victor, 1967; O'Leary et al., 1968, 1971; Murphy et al., 1973; Fox et al., 1969; Chan-Palay and Palay, 1970, 1971; Palay and Chan-Palay, 1974; Rivera-Domínguez et al., 1974) have provided the basic morphological aspects of climbing fibers as reported almost one hundred years ago, in the pioneering and classical account of Ramón y Cajal (1888, 1955).

In previous papers we have briefly described, by means of conventional scanning electron microscopy (SEM), ethanol-cryofracturing technique and the freeze-fracture method for SEM, the course of climbing fibers through the granule cell, Purkinje cell and molecular layers of mouse, rat, fish and human cerebellum (Castejón and Caraballo, 1980a,b; Castejón and Valero, 1980; Castejón, 1983, 1986, 1988). In such studies, the depth of focus of SEM allowed us to observe and identify, in a preliminary way, the parent climbing fibers and their pattern of bifurcation. The formation of tendril collaterals and climbing fiber glomeruli in the granule cell layer was also reported. In addition, at the Purkinje cell and molecular layers, the relationship between climbing fibers and Purkinje dendritic arborization was also seen.

Further studies have been carried out in our laboratory in order to characterize by the SEM-freeze fracture method, the climbing fiber synaptic relationship with the Purkinje spiny branches in fish cerebellar molecular layer and to obtain new and complementary information by using the freeze-etching technique applied to mouse cerebellar cortex.

Using the freeze-etching technique, Landis and Reese (1974) and García-Segura (1985) have reported some synaptic intramembrane features of the climbing fiber-Purkinje dendrite system, which have been correlated with other central excitatory synapses. These findings support the notion as earlier pointed out by Eccles et al. (1967), that the climbing fiber-Purkinje spine synapse represents one of the most powerful excitatory synaptic systems of cerebellum.

In earlier electron microscope cytochemical

studies (Castejón and Castejón, 1972a, b,c) we have reported in other excitatory synapses, the mossy fiber-granule cell synapses of mouse cerebellar glomeruli, an increase of electron density at the pre-synaptic axoplasm, in the pre- and post-synaptic densities and also in the synaptic cleft, when two basic electron stains, the Alcian Blue and the osmium coordination compound, Os-DMEDA, were applied. After various enzymatic digestions (Castejón and Castejón, 1976), some of these findings were correlated with the presence of synaptic polyanionic compounds such as glycosaminoglycans. These compounds joined to a protein core, constitute the proteoglycans which, beside glycoproteins, have acquired an increasing interest during the last years. Several reports have been published dealing with their metabolism in neurons and particularly with the parameters of axoplasmic transport and mechanisms of synaptic transmission. (For review, see Margolis and Margolis, 1979).

In view of the increasing functional importance of these compounds and also of the climbing fiber-Purkinje spine system, we have considered it of great interest first, to go deeper into the submicroscopic details of this synaptic system, and second, to initiate its cytochemical study by applying some electron stains to cerebellar tissue, in the hope of getting an insight into its chemical characteristics. Thus, in the present paper, we will report the SEM three dimensional morphology (fish cerebellum) and freeze-fracture features (mouse cerebellum) of cerebellar climbing fibers, besides the preliminary cytochemical results, obtained in mouse cerebellum with two electron stains, Alcian Blue-OsO_4 and $RuCl_3$.

Materials and Methods

Freeze-fracture scanning electron microscopy

Specimens of two teleost fishes: Arius spixii and Salmo trutta were fixed by vascular perfusion with Karnovsky fixative. Cerebellar slices, 2-3mm thick, were cut with a razor blade and fixed by immersion in the same fixative for 4-5h. After washing in buffered saline, they were post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer solution, pH 7.4 for 1h. After rinsing in a similar buffer, tissue blocks were dehydrated through graded concentrations of ethanol, rapidly frozen by plunging into Freon 22, cooled by liquid nitrogen (Haggis and Phipps-Todd, 1977) and fractured with a precooled razor blade. The fracture fragments were returned to fresh absolute ethanol for thawing. The tissue was then dried by the critical point method with liguid CO_2 as recommended by Anderson (1951) and coated with gold-palladium. Specimens were examined in a JEOL 100 B electron microscope with ASID scanning attachment at 80kV.

Electron microscope cytochemical study

Alcian Blue staining. For this purpose, male or female adult normal albino mice were used. A group of mice were processed according to the Benhke and Zelander (1970) perfusion 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 similarly 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, 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 post-fixed 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 post-fixed in osmium tetroxide without previous Alcian Blue treatment, and processed for electron microscopy as described above. Another 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 has been added. After washing in a similar buffer, the tissues were immersed in 0.1% OsO_4-0.1M cacodylate buffer at pH 6.5 for 2h, dehydrated and embedded in Araldite. For enzymatic digestion parallel slices of glutaraldehyde fixed cerebella, after washing in buffer phosphate pH 7.4, were incubated in 0.05% bovine testicular hyaluronidase (Sigma Type V) in 0.1M phosphate buffer pH 5.5 for 3h at 37°C. All ultrathin sections were stained with uranyl acetate and lead citrate and examined in the electron microscope.

Ruthenium Chloride Staining. Albino mice were perfused with a solution of 1.25% glutaraldehyde in 0.1M cacodylate buffer pH 7.4, to which 5% sucrose and 0.1% ruthenium chloride were added. After 20-30 minutes perfusion, the brain was removed and the cerebellar cortex was diced into small blocks. They were further fixed by immersion in a fresh similar mixture for 1h and subsequently immersed, for 2h in the dark, in a 0.5% osmium tetroxide-cacodylate-sucrose buffer solution to which 0.05% ruthenium chloride was added. Some cerebellar pieces continued fixation for the same time in 0.05% ruthenium chloride without OsO_4 addition. After dehydrating and embedding in Araldite, ultrathin sections were stained by uranyl acetate and lead citrate. Controls consisted of uranyl and lead stained sections of aldehyde perfused cerebellar tissue, with and without OsO_4 post-fixation.

Freeze-etching and direct replicas. Adult Swiss albino mice were injected intraperitoneally with Nembutal (Sodium pentobarbital) and sacrificed by decapitation. The brains were carefully removed and 1-2mm slices of cerebellar cortex were fixed in 1% ice cold glutaraldehyde-0.1M phosphate buffer, pH 7.2-7.4 for 1h. The slices were then 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 -100°C, in vacuum of 4×10^{-6}. Fractured surfaces were shadowed with a layer of carbon platinum about 2.5nm thick. Replicas were floated off on water, bleached in chlorox overnight, rinsed in water, bathed in 50% H_2SO_4 and rinsed in several changes of water. Cleaned replicas were mounted on grids usually coated with Parlodion or formvar films, and examined with a JEOL 100 B electron microscope.

Results

Identification criteria

The following criteria have been taken into account for the characterization of the climbing fibers at scanning electron microscope level:

Intracortical course. Due to the large depth of focus of scanning electron microscope and the panoramic view obtained with sagittal fractographs of cerebellar cortex examined at low magnification, it is possible to visualize the complete traject of climbing fibers through the granular layer, their typical topographic relationship with Purkinje cell body and dendritic arborization and their terminal ramifications in the molecular layer. On the contrary most mossy fibers in general remain in the granular layer. The third type of the cerebellar afferents, the monoaminergic fibers (noradrenergic and serotoninergic fibers) terminates in both the granular and molecular layers. However, at the present time the identification of these latter fibers cannot be done by SEM morphological features but by biochemical criteria only.

Branching pattern. Climbing fibers show a typical cross-over or arborescence pattern type of bifurcation (Castejón, 1983). Their ascending and descending collaterals characteristically spread out in three different planes. On the other hand the mossy fibers generally arborize dichotomously.

Fiber thickness. In the granular layer the climbing fibers are usually thinner than mossy fiber (Mugnaini, 1972) while in the molecular layer they are thicker than the granule cell axons.

Type of synaptic junctions. In the granular layer climbing fibers form terminal glomerular synapses. Conversely the mossy fibers make "en passant" glomerular synapses (Castejón and Caraballo, 1980b, Castejón, 1988). These features allow us to distinguish at SEM level mossy fiber glomeruli from climbing fiber glomeruli. In the molecular layer climbing fibers and parallel fibers establish spine synapses with the Purkinje dendritic arborization, but the climbing fibers synapse mainly with large Purkinje dendritic spines. With low SEM resolution and SEM freeze-fracture method until now we have not been able to visualize spine synapses. They are better characterized, as will be shown in the present study, by transmission electron microscopy and freeze-etching technique. Serotoninergic fibers only rarely exhibit junctional complexes whereas noradrenergic fibers make synaptic contacts with major Purkinje dendrites and with small dendritic spines (Chan-Palay, 1975). Therefore, until now monoaminergic fibers have been difficult to characterize by SEM methods due not only to the low SEM resolution but to the absence of a combined SEM morphological and biochemical technique.

Freeze-fracture scanning electron microscopy

The climbing fibers appeared in sagittally orientated fractographs of cerebellar cortex as large thick axons crossing the granule cell layer. Two different types of collaterals, as described by Palay and Chan-Palay (1974), were emitted from the parent climbing fiber during its course through this layer: the fine tendril collaterals (Fig. 1), characterized by varicosities or globular enlargements connected by a fine thread, closely resembling the Golgi cell axonal ramifications, and the second type or thick collaterals (Palay's glomerular collaterals), which maintain roughly the same calibre as the parent stem and end in a truncated spray or large efflorescence. The latter ones, as the mossy fibers, could be seen participating in the formation of glomeruli. The climbing fiber glomeruli appeared as thin elongated structures, where the large efflorescences of glomerular collaterals were surrounded by granule cell dendritic digits and could be clearly differentiated from the mossy fiber glomeruli, which are larger and round or polygonal. The climbing fiber glomerular collaterals form terminal glomerular synapses whereas mossy fibers form "en passant" glomerular synapses.

After crossing through the granular layer, the parent climbing fibers were observed approaching the Purkinje cell soma (Fig. 2) and then ascending toward the surface of the folium closely applied to the Purkinje primary trunk and secondary dendritic branches. At the level of the molecular layer, the climbing fibers emitted fine beaded branches about 0.1µm in diameter, which characteristically spreaded out in three different planes (Fig. 3). The terminal arborization of climbing fibers was seen ending by means of fine tendrils on the Purkinje spiny dendritic branches (Fig. 4). The low SEM resolution and the plane of the fractographs limit the visualization of climbing fiber spine synapses, which are better studied three-dimensionally by freeze-etching technique. The fracture process also limits the visualization of the retrograde collaterals of climbing fibers going from the molecular layer to the granular layer.

Ultracytochemical study

For this study, only those varicosities which appeared parallel to the main primary and secondary Purkinje dendrites and making contact with more than two Purkinje spines were considered to be climbing fiber pre-synaptic terminals (Fig. 5). These round or elongated pre-synaptic endings, originating from the climbing fiber tendrils and making contact with large and stubby Purkinje dendritic spines were characterized by a dense packing of spheroidal synaptic vesicles. A dense axoplasmic material surrounding the synaptic vesicles was observed in the tissue fixed, either by vascular perfusion or by immersion, with glutaraldehyde-Alcian Blue mixture, postfixed in osmium tetroxide and the thin sections post-stained with uranyl acetate and lead citrate. In addition, a remarkable staining of the pre- and post-synaptic densities and also of the intracleft material was

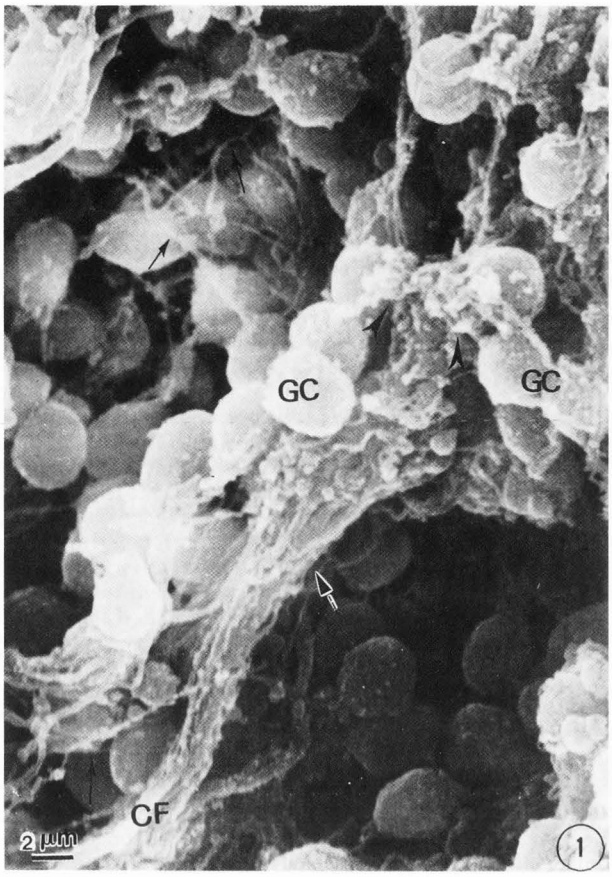


Fig. 1. Teleost fish cerebellum. Granular layer. Parent thick climbing fibers (CF) showing the fine tendril collaterals (thin arrows) and the glomerular collaterals (short arrow). The latter ones penetrate into a granule cell group (GC) forming a climbing fiber glomerulus, which appears partially fractured. The arrowheads point out the synaptic relationship between granule cell dendrites and the glomerular collaterals.

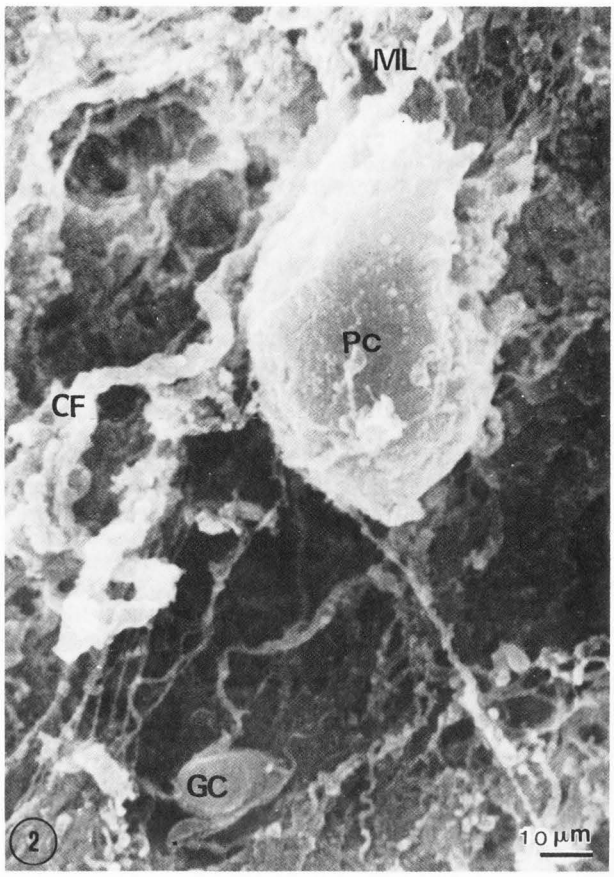


Fig. 2. Teleost fish cerebellum. Purkinje cell layer. The climbing fiber (CF) emerges from the granular layer and then approaches the Purkinje cell soma (Pc) ascending toward the molecular layer (ML). A granule cell (GC) is observed at the bottom of the fractograph.

obtained. Hyaluronidase treatment removed stainable material from axoplasm but left pre- and postsynaptic densities intact (Fig. 6).

The application of $RuCl_3$ to mouse cerebellar tissue led to the following observations: The glutaraldehyde-$RuCl_3$ perfusate solution showed a clear light yellowish coloration. Its tissular penetration and impregnation was apparently faster, since the fairly hard cerebellar consistency after perfusion permitted easy hand sectioning. After osmication, $RuCl_3$ impregnated blocks showed a light brownish shade, while control glutaraldehyde perfused cerebellar tissue appeared, as expected, completely darkened. In addition, in $RuCl_3$ treated nerve tissue, with and without osmication, the staining with uranyl and lead salts failed to show contrast at cell membranes. These two separate observations appear to be indicative of osmication blocking by $RuCl_3$ although impregnation was done in darkness. On the other hand, in $RuCl_3$ impregnated tissue, a maximal contrast of synaptic paramembranous elements and intracleft material was achieved. At climbing fiber-Purkinje spine synapses (Fig. 7), a ruthenium stained axoplasmic dense substance was seen surrounding the synaptic vesicles and continuous with the presynaptic dense projection. Synaptic vesicles were recognized by their spheroidal shape and location at the presynaptic active zone whereas no stainable intravesicular material was detected. At the synaptic cleft, two layers of dense substance separated by a narrow gap 2nm in width, were observed. An enhancement of postsynaptic density was also found.

Freeze-etching study

In freeze-fracture replicas of mouse cerebellar molecular layer fractured in the parasagittal planes (Figs. 8 and 12), the climbing fiber endings were positively identified by: a) their larger size in comparison with the parallel fiber endings; b) a dense packing of synaptic vesicles and c) the formation of axospinodendritic contacts with the large Purkinje dendritic spines. In such replicas, the climbing fiber varicosities were well displayed and appeared fractured in a plane parallel to the main axis of large Purkinje

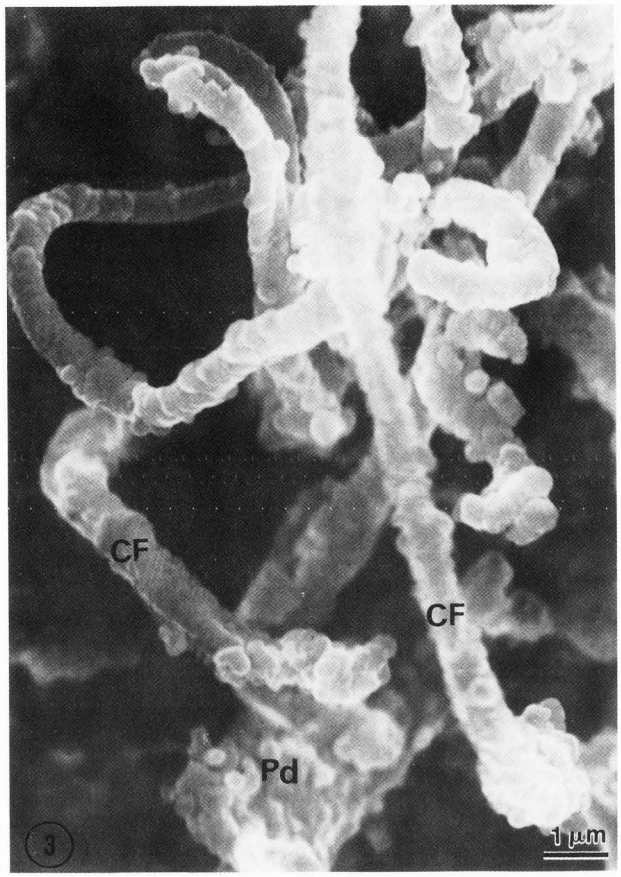


Fig. 3. Teleost fish cerebellum. Molecular layer. Climbing fibers (CF) showing the typical cross-over or arborescence pattern type of bifurcation, characteristically spreading in three different planes. A Purkinje dendrite (Pd) is observed in the close neighbourhood. The plane of the fractograph limits the visualization of Purkinje spines.

dendrites. In the close neighbourhood, the cross sections of synaptic and non-synaptic segments of parallel fibers were also disclosed. When the fracture plane occurred parallel to the plane of the synaptic cleft, a large surface view of the internal face of the outer leaflet (E-face) of climbing fiber presynaptic membrane was obtained, as seen from an intracellular vantage point (Fig. 9). The examination of this surface revealed elevated localized patches, which were correlated with active synaptic sites. They were characterized by clusters of intramembrane particles (IMPs), 3 to 5 randomly distributed membrane protuberances and pits or craters, with diameters of approximately 12-16 nm. The elevated patches appeared surrounded by IMPs free membrane domains. At these regions, the fracture process usually continued by splitting the Purkinje spine postsynaptic membrane, exposing its P face and finally extending toward the enveloping Bergmann glial cell, which disclosed its P face and cytoplasm. The P face Purkinje spine membrane was characterized by a high density distribution of IMPs ranging in diameter 5.70 to 10.74nm.

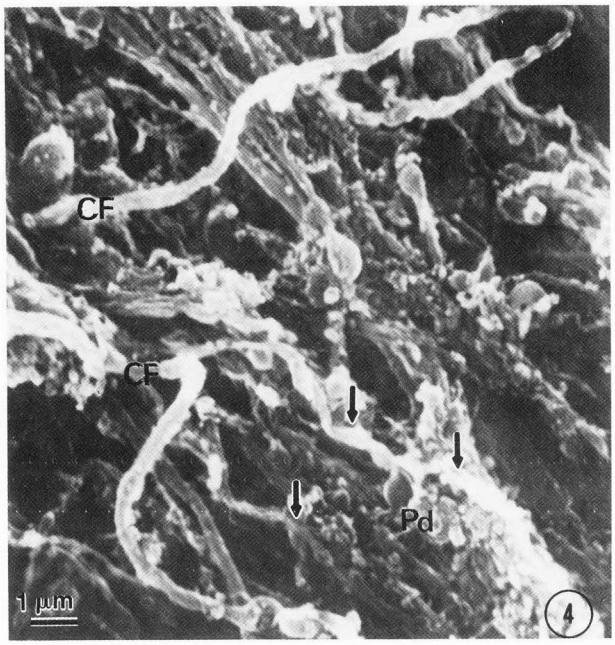


Fig. 4. Teleost fish cerebellum. Outer third of the molecular layer. Climbing fibers (CF) and their terminal arborization in the molecular layer. The fine terminal ramifications of climbing fibers (arrows) end as fine beaded processes over the surface of Purkinje dendritic branchlets (Pd).

Usually the climbing fiber endings contact with several Purkinje spines. In these multiple thorn synapses, synaptic contacts were mainly established with the bulbous tip of the spine. In addition, lateral contacts with the stalks and body of the spines were also observed (Fig. 10). In such areas the freeze-fracture procedure exposed the cross-fractured climbing fiber endings containing numerous spheroidal synaptic vesicles and the P face of the postsynaptic membrane of the Purkinje spine.

In tangentially fractured climbing fiber endings, the fracture plane splitted the presynaptic cluster of vesicles and subsequently shifted into the presynaptic membrane, exposing the P face at the active zone (Fig. 11). At this region, aggregates of intramembrane particles and exocytosis vesicle sites occurred in register with the synaptic vesicles cluster. Three types of intramembrane particles (IMPs) were distinguished: small IMPs = 3.69-5.95nm, medium IMPs = 6.19-8.92nm and large IMPs = 9.04-12.50nm, which were found scattered throughout the P face or in clusters.

When the fracture plane occurred through the center or the equatorial plane of the climbing fiber varicosity, there was exposure of a large area of the site of the junction between the climbing fiber ending and the Purkinje spine (Fig. 12). The synaptic cleft between both structures was clearly widened and the apposed pre- and postsynaptic membranes were fractured at right angles. A linear aggregate of intramembrane particles was often seen in the external half (E-face) of the postsynaptic spine membrane.

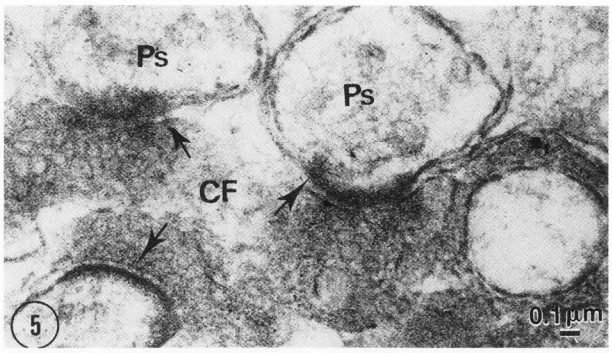


Fig. 5. Mouse cerebellar cortex. Molecular layer. Glutaraldehyde-Alcian Blue perfused cerebellum. Climbing fiber ending (CF) making a multiple thorn synapse (arrows) with Purkinje dendritic spine (Ps). The climbing fiber axoplasmic matrix shows an Alcianophylic electron dense extravesicular material. The synaptic complexes exhibit an increased electron density.

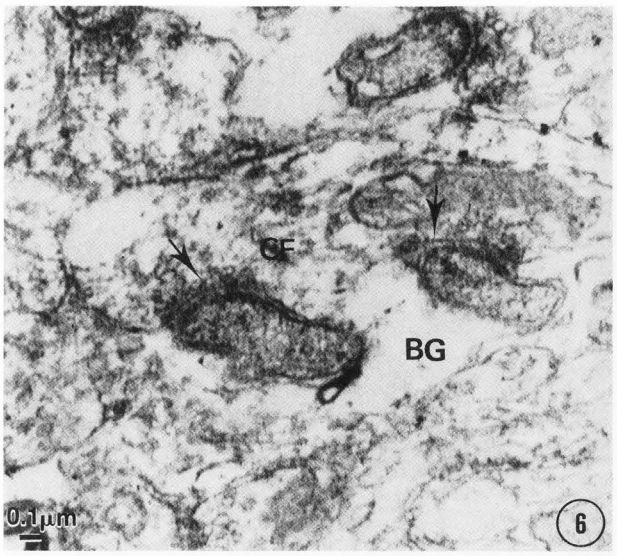
Discussion

The characterization of climbing fibers was made in accordance with their morphology, size, branching pattern, intracortical course and synaptic relationship. The SEM appearance of climbing fibers can be well correlated with light microscope studies carried out by Ramón y Cajal (1888), Estable (1923), Scheibel and Scheibel (1954) and the light and electron microscopic descriptions reported by Hamori and Szentagothai (1966) and by Palay and Chan-Palay (1974).

A distinctive feature of SEM climbing fiber morphology was the cross-over bifurcation pattern in the granular and molecular layers (Castejón, 1983, 1988). This characteristic feature was not observed in mossy fiber, stellate or basket cell axons. The cross-over which follows branching was first described by Athias (1897) and more recently by O'Leary et al. (1971).

As Ramón y Cajal (1955) has shown, the climbing fiber increases in complexity as the phylogenetic scale is ascended. However, the basic structure and connections remain the same in all the species (Scheibel and Scheibel, 1954). The morphological separability of climbing fibers from other axonal substrates and the cross-over which follows branching as described in the present paper, are features which have also been observed by O'Leary et al. (1971) during the histogenesis of the rat cerebellar climbing fibers.

With SEM it is possible to estimate with a more reliable degree of certainty the amount of branching of climbing fibers in the granular and molecular layers. This subject has been a matter of controversy in the past. Ramón y Cajal (1955) did not describe climbing fiber collaterals in the granular layer. They were first described by Scheibel and Scheibel (1954) as retrograde collaterals descending from the molecular layer. More recently a detailed account of climbing fiber bifurcation



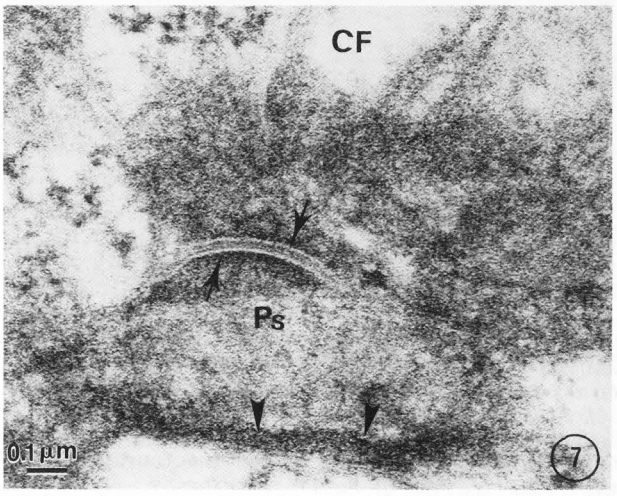


Fig. 6. Mouse cerebellar cortex. Molecular layer. Effect of testicular hyaluronidase treatment on glutaraldehyde fixed-climbing fiber terminal, followed by Alcian Blue staining. The enzymatic treatment almost completely degraded the electron dense material present in the climbing fiber varicosity (CF). Pre- and post-synaptic densities (arrows) appear also partially degraded. The electron lucent Bergmann glial process (BG) encapsulates the climbing fiber spine synapses. The enzymatic digestion alters optimal preservation of ultrastructural features.

Fig. 7. Mouse cerebellum. Ruthenium chloride staining. Climbing fiber-Purkinje spine synaptic contact. The climbing fiber ending (CF) exhibits an electron dense substance surrounding the synaptic vesicles. A double dense layer separated by a clear interspace is observed within the synaptic cleft. The pre- and post-synaptic dense projections (arrows) show an enhanced electron density. A paramembranous material (arrowheads) is also stained in the Purkinje spine (Ps).

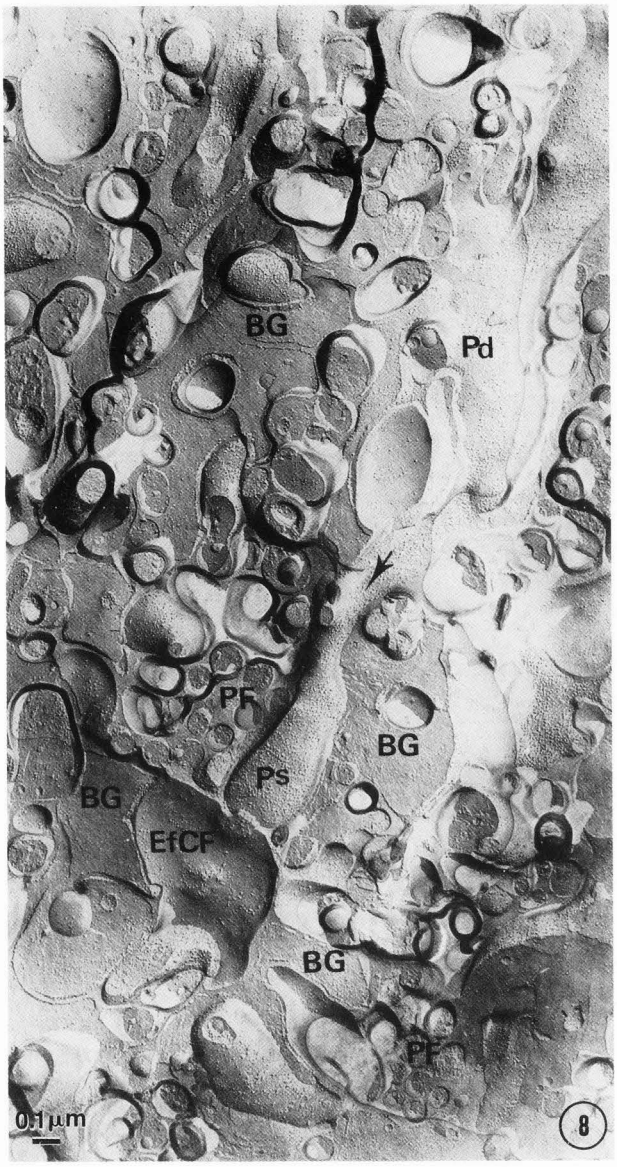


Fig. 8. Mouse cerebellar molecular layer fractured in the sagittal plane revealing the P face of a longitudinally fractured Purkinje dendrite (Pd) and its spine (arrow, Ps). The plane of the fracture has exposed the external half of the presynaptic membrane of a climbing fiber bouton (EfCF) synapsing with the Purkinje spine (Ps). The cross section of parallel fiber bundles (PF) and the fractured cytoplasm of Bergmann glial cell processes (BG) are also observed.

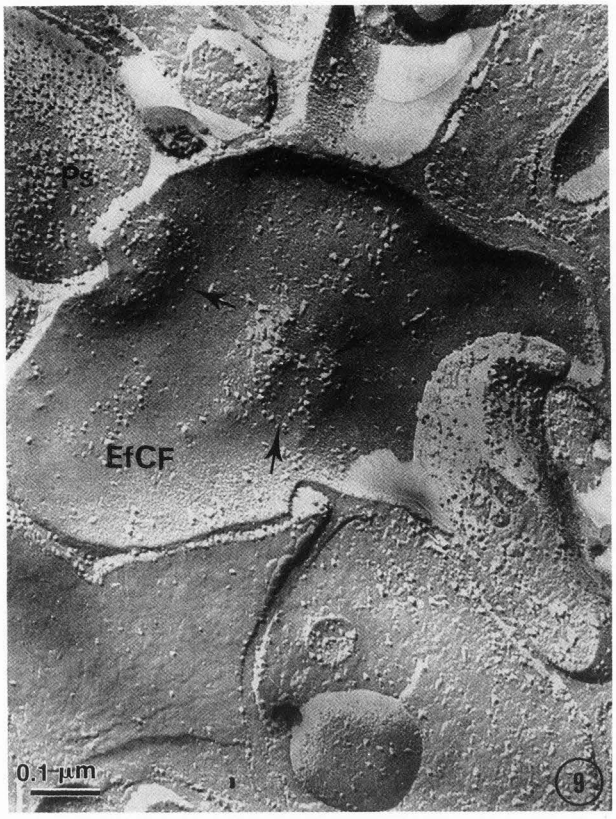
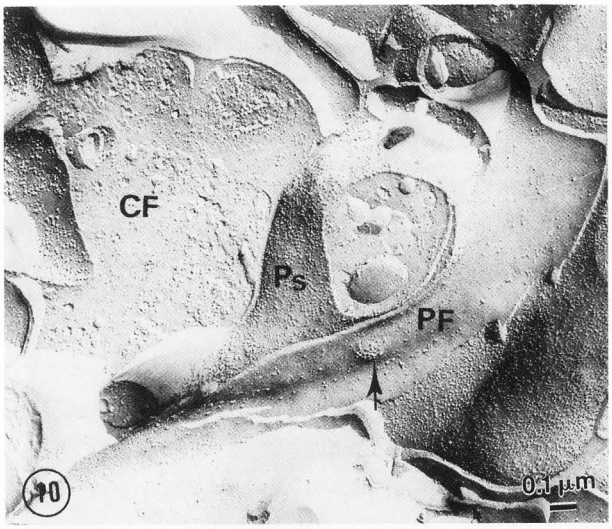


Fig. 9. Higher magnification of the climbing fiber-Purkinje spine (Ps) synapse illustrated in figure 8. The E face of the fractured pre-synaptic climbing fiber membrane can be observed as seen from inside the terminal (EfCF). At two synaptic active sites some protuberances and depressions (arrows) are appreciated, surrounded by clusters of intramembrane particles. The plane of fracture has also exposed the P face of Purkinje spine post-synaptic membrane showing the high density distribution of IMPs.

in the granular layer was made by Fox et al.(1969) and Chan-Palay and Palay (1971). In the present study a higher incidence of branching of climbing fiber was detected in the granular layer, especially at the level of tendril collaterals between the granule cell groups.

In addition, SEM offers the advantages of following the tortuous course and extreme tenuity of tendril collaterals in the molecular layer, overcoming the inherent difficulties derived from the interpretation of TEM fine section studies. Larramendi and Victor (1967) showed that climbing fibers synapse on thorns emitted from the so-called smooth branches of the Purkinje dendritic tree and not directly upon the dendritic shaft according to the classical descriptions of Ramón y Cajal (1955). The SEM observations of climbing fibers at the molecular layer clearly showed that these fibers are not really attached to the Purkinje dendritic trees but separated by an interspace, supporting the Larramendi and Victor (1967) TEM study.

The study of climbing fibers in the molecular layer has been facilitated by the selective removal of Bergmann glial cell by the cryofracture process. This neuroglial covering was mentioned by Hamori and Szentagothai (1964) as an obstacle for



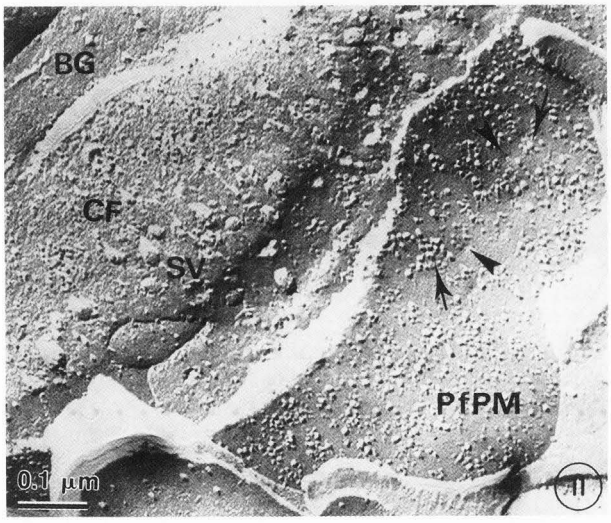


Fig. 10. Cross fractured climbing fiber synaptic knob (CF) attached to the lateral side of the stalk and bulbous body of Purkinje spine (Ps). The E face of a parallel fiber (PF) varicosity is observed in the opposite side of the spine showing IMPs aggregation at the pre-synaptic active zone (arrow).

Fig. 11. Tangentially fractured climbing fiber ending (CF) showing synaptic vesicles (SV) in the fractured cytoplasm. Aggregates of intramembrane particles (arrows) and exocytosis vesicle sites (arrowheads) are observed at the P face pre-synaptic membrane (PfPM). Large intramembrane particles appear scattered among the medium sized IMPs aggregates. The cytoplasm of Bergmann (BG) glial cell process is observed in the upper left corner of the figure.

visualizing climbing fibers at that level. In both the granular and molecular layers, we searched for axo-axonic connections of climbing fibers with mossy fibers, stellate and basket cell axons and

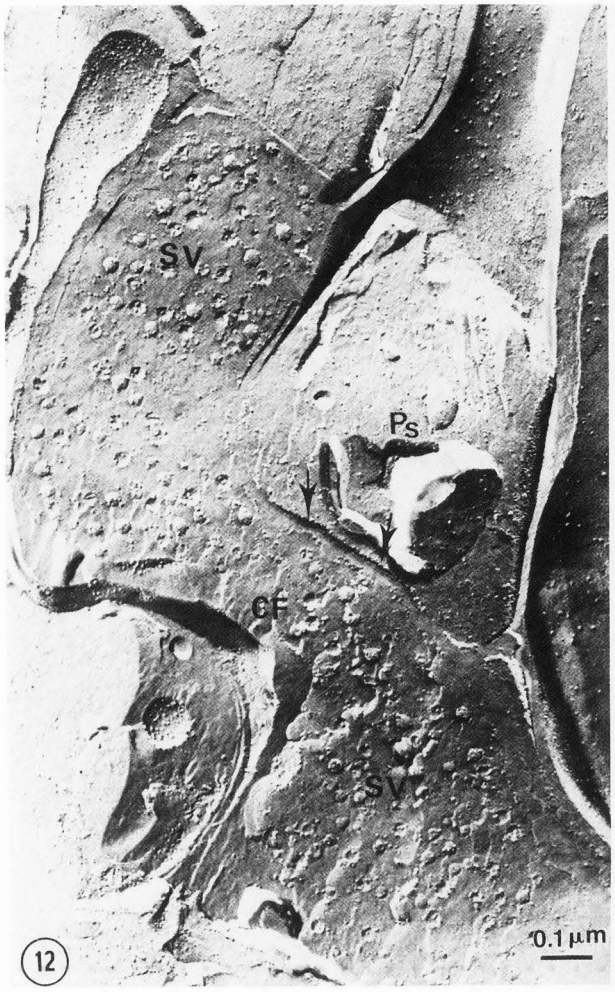


Fig. 12. A longitudinally fractured large climbing fiber varicosity (CF) contains numerous spheroidal synaptic vesicles (SV) and synapse with a Purkinje spine (Ps). The Purkinje spine has been partially fractured exposing rows of intramembrane particles (arrows) at the post-synaptic membrane.

parallel fibers. The existence of this kind of synapses has been mentioned by Scheibel and Scheibel (1954). However, until now we have not found evidence of such axo-axonic contacts. TEM studies (Chan-Palay and Palay, 1970) have also failed to reveal such specialized contacts. Nearly all the synaptic contacts made by climbing fiber tendril varicosities are upon Purkinje spines. As previously postulated by Palay and Chan-Palay (1974), "this form of synaptic contact appears to be favorable for the rapid conduction of an impulse over a long distance with almost simultaneous dispersal to multiple sites of transmission. The arrangement of this extensive synaptic apparatus seems to be a device for achieving a very high efficiency of activation of the Purkinje dendritic tree".

The SEM image of climbing fiber arborization

in the molecular layer, agrees with the properties of the climbing fiber-Purkinje cell system as described in electrophysiological studies (Eccles et al. 1967). However, since the climbing fibers activate through their varied connections in the granular and molecular layers, both excitatory and inhibitory pathways leading to the Purkinje cell, it becomes a difficult and complicated undertaking to correlate the morphological findings with the interpretation of physiological records (Palay and Chan-Palay, 1974). Our study also confirms that each varicosity in a climbing fiber usually interacts with several thorns. The multiple thorny climbing fiber synapses, as illustrated in figure 5, suggests that the excitation of climbing fiber would discharge a great deal of transmitters into the synaptic cleft and activate all the attached thorns simultaneously. As has been previously expressed by Palay and Chan-Palay (1974), "Since the thorns are clustered together, the small amount of current injected into the dendrite from each thorn would produce a small postsynaptic potential that would summate first with the small postsynaptic potentials arising from the other thorns in the cluster. Spreading electrotonically, this potential would then summate with others arising from similar thorn clusters that were activated at almost the same moment along the path of the climbing fibers".

Ultracytochemical study

This study has been initiated in order to test the probable glycosaminoglycan content of climbing fiber presynaptic grid. The staining mechanism of Alcian Blue as electron cytochemical stain for revealing acidic compounds has been already reported (Tice and Barnett, 1962; Behnke and Zelander, 1970; Castejón and Castejón, 1972a, b; 1976; Schofield et al. 1975; Ruggeri et al. 1975). This staining technique is not expected to be specific for glycosaminoglycans since it stains any polyanionic macromolecule including sialic acid glycoproteins, sulfated glycoproteins, some gangliosides and also nucleic acids. The electron dense substance observed at the climbing fiber presynaptic grid, axoplasmic matrix around synaptic vesicles, pre- and postsynaptic densities and synaptic cleft, should be constituted by one or several polyanionic macromolecules such as glycosaminoglycans or acidic glycoproteins. Enzymatic digestion studies could provide additional information; so, the previous application of enzymes such as hyaluronidases, chondroitinases and neuraminidases are required for a proper identification of polyanions. We have applied testicular hyaluronidase treatment which removed stainable material from axoplasm. Since the hyaluronic acid, chondroitin 4-sulphate and chondroitin 6-sulphate are the major natural substrates of the enzyme, we infer that the predominantly stained substance of climbing fiber presynaptic axoplasm is constituted by proteoglycans in which one or several of the before mentioned glycosaminoglycans are present. An axoplasmic and presynaptic substance with similar characteristics has been previously reported by us in mossy fiber presynaptic endings, which was also partially sensitive to carboxymethylation and testicular hyaluronidase and presumed to be glycosaminoglycans. However, results with hyaluronidase must be regarded as supportive rather than conclusive proof of identification, because this procedure may produce destruction of specific substrates other than glycosaminoglycans, or non-specific destruction caused by contaminant glycosidases and proteases in the enzyme preparations. Recently, Aquino and Margolis (1984) applied some immunocytochemical techniques to nerve tissue for revealing chondroitin sulphate proteoglycan and demonstrated also a positive axoplasmic staining in another excitatory cerebellar system, the parallel fiber-Purkinje cell circuit.

With respect to $RuCl_3$ stain, its mechanism of staining is unknown. The results obtained by the application of this stain on nerve tissue led to rather contradictory observations since it behaves more similarly to the bismuth iodide (BIUL) method (Pfenninger, 1971a,b) than to ruthenium red staining (Tani and Ametani, 1971). The latter is presumed to reveal anionic macromolecules as glycosaminoglycans and it requires osmium tetroxide to increase electron density. With this staining an electron dense substance at the extracellular and intracellular spaces is observed. However, because of the poor penetration of the dye, the staining properties of intracellular structures is yet unknown in detail. On the contrary our $RuCl_3$ pre-embedding staining method showed a faster tissular penetration and despite its failing to stain membrane structures it gave positive electron density to the paramembranous elements, such as the axoplasmic matrix surrounding synaptic vesicles, the presynaptic grid and the pre- and also the post-synaptic densities. These results appear to be similar to those reported with other staining techniques for acidic groups (Kuriyama and Okada, 1971; Castejón and Castejón, 1972a,b, 1976) including the BIUL method (Pfenninger, 1971a, b). The latter method is supposed to reveal both basic amino residues and anionic groups, by forming a complex on one side with tissular basic groups and on the other side with uranyl and lead which bind to tissular anionic groups. At the synaptic cleft, the application of $RuCl_3$ method showed two external fuzzy coats separated by a gap of 2nm. This finding appears to be similar to that reported in BIUL treated synapses, by Pfenninger (1971a,b) who also pointed out that the gap, which is stainable by other methods, may contain a cleft substance bearing polar groups possibly bound in such a way that inhibit the staining with the respective contrasting agents. This binding could represent a polyionic binding mechanism of synaptic connectivity (Pfenninger, 1971b), since earlier reports of Wolfe (1961) and also Bennedetti and Emmelot (1967) have demonstrated binding of basic proteins to synaptosome and membrane fractions, respectively. However, further experiments will be required to properly correlate BIUL and $RuCl_3$ similarities. As mentioned above, the various staining techniques applied to nerve tissue for revealing acid glycosaminoglycans by electron microscopy fail to be specific since they would be expected to stain any polyanionic macromolecule. Also, the newer immunocytochemical techniques for revealing proteoglycans (Aquino and Margolis, 1984; Bignami and Dahl, 1986) fail to reveal each of the different proteoglycans present in nerve tissue, because of

the actual limitation in obtaining specific antibodies. On the other hand, the quality of enzymes for biochemical tests is improving each day. We consider therefore, that the application of enzymes in conjunction with the available staining techniques for polyanions are still of a great value for the demonstration of glycosaminoglycans in the nerve tissue.

The study of glycosaminoglycans at synapses has been of great interest since these acidic compounds seem to play a role in synaptic functions: either as a buffer, controlling the storage and release of transmitter in synaptic vesicles and extracellular space; or maintaining the electrical stability due to their ability to bind calcium or absorb water; or by limiting the concentration of potassium ion in the extracellular space by binding it or buffering its action (for references see Margolis and Margolis, 1977,1979; Brunngraber, 1979). The apparently selective presence of certain types of glycosaminoglycans in the axoplasm of three cerebellar excitatory systems would rather indicate that these polyanions are involved in some excitatory mechanisms of neurotransmission. Synaptic glycoproteins, besides their importance in connectivity and cell to cell recognition may act as trophic factors, transported and released by climbing fibers, serving to modulate the expression of a specific membrane component of Purkinje cell (García-Segura, 1985).

Freeze-etching observations

Because of its ability to disclose protein moieties at the synaptic membranes, the freeze-etching technique permitted the visualization of internal details of a typical excitatory synapses such as the climbing fiber-Purkinje spine synapses. An aggregation of intramembrane particles at the E face presynaptic membrane as well as vesicles attachment sites were seen. These membrane features are in accordance with the characteristic presynaptic specialization at excitatory synapses (Pfenninger et al. 1972; Sandri et al. 1972; Landis and Reese, 1974; Tokunaga, 1979; Korte and Rosenbluth, 1980; Hama, 1980; Raviola and Raviola, 1982; García-Segura and Perrelet, 1982, 1984). The P face presynaptic membrane particles have been correlated with calcium ionophores and the postsynaptic aggregates of intramembrane particles seem to represent the distribution of transmitter receptor proteins, such as the acetylcholine receptor protein (Venzin et al. 1977; Sandri et al. 1972; Rash and Ellisman, 1974; Heuser and Salpeter, 1979). The presence of protuberances or pits on the E face of the climbing fiber presynaptic membrane would correspond to attachment sites between synaptic vesicles and presynaptic membrane or transmembranous molecular channels (Pfenninger et al. 1972). As illustrated in figures 8 and 9 a high distribution of IMPs was found in the P face Purkinje spine postsynaptic membrane. Glutamate and aspartate have been tentatively postulated as neurotransmitter candidates of climbing fibers (Kanazawa, 1986). In this context some intramembrane particles shown at the Purkinje spine postsynaptic membrane may morphologically represent the glutamate or aspartate receptor-ion channels complexes. The postsynaptic density observed in thin sections probably corresponds to the region of membrane that contains the intramembrane particle aggregates observed in freeze-fracture replicas (Pfenninger el al. 1972; Sandri et al. 1972; Landis and Reese, 1974; Korte and Rosenbluth, 1980; Gulley and Reese, 1981).

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Discussion with Reviewers

Reviewer I: What criteria were used to identify cells? Location? Orientation? Associations?
Authors: In previous papers we have analyzed in detail the SEM cytoarchitectonic arrangement of vertebrate cerebellar cortex (Castejón and Caraballo, 1980b; Castejón and Valero, 1980; Castejón, 1983; Castejón, 1986). In such studies the SEM fractographs have been correlated with light microscopic observations, transmission electron microscopy and freeze-etching replicas. In a recent publication (Castejón, 1988) we have given the criteria for identification of granule cell, Purkinje cell and molecular cell layers. The cerebellar cortex is a well studied, highly organized area of the brain containing five closely packed major neuronal types (granule cells, Golgi cells, Purkinje cells, stellate and basket cells), stratified in three distinct, geometrically arranged layers. The slicing technique for SEM, ethanol-cryofractography for SEM, freeze-fracture method for SEM and freeze-etching replicas have been used in conjunction to study the three-dimensional arrangement of each layer of vertebrate cerebellar cortex. The results obtained have been compared with light and transmission electron microscope findings in order to achieve proper identification of cerebellar microneurons (granule, stellate and basket cells) and macroneurons (Golgi and Purkinje cells). In addition the intracortical circuits formed by afferent fibers (mossy and climbing fibers) with the intrinsic fibers have been traced by SEM techniques.

Reviewer I: The image quality in Fig. 6 does not allow this reviewer to accept the statements concerning enzymatic digestion of extracellular materials, or to accept any conclusions concerning the extracellular location of any specific matrix components.
Authors: In the present study we are mainly dealing with the glycosaminoglycan content within the axoplasmic climbing fiber and not with the extracellular matrix components. Figs. 5, 6 and 7 tend to demonstrate the presence of hyaluronic acid, chondroitin 4-sulfate and chondroitin 6-sulfate at the level of the presynaptic grid of climbing fiber endings.

Reviewer I: The freeze-fracture micrographs suffer from the absence of low magnification "overviews". The authors should list the criteria used to identify specific cell processes.
Authors: Fig. 8 is a low magnification overview of cerebellar molecular layer. The criteria for identification of specific cell processes are given in the results under the heading: Identification criteria. The reader is referred to the review published by one of us (Castejón, 1988) for a more detailed explanation of identification criteria for afferent fibers (mossy fiber and climbing fibers) and intrinsic fibers (parallel fibers, stellate, basket, Golgi and Purkinje cell axons).

Reviewer I: How was the Bergmann glial fibers identified?
Authors: Bergmann glial cells are regional types of cerebellar astrocytes, satellites of Purkinje cells. At the transmission electron microscope level they exhibit a clear cytoplasm. Their ascending processes or Bergmann glial fibers surround the Purkinje primary dendritic trunk and accompany the secondary branches and tertiary dendritic branchlets in the molecular layer. This topographic relationship allows their identification in the molecular layer. Bergmann glial fibers form lamellated processes, which can be easily traced in the complex neuropil of cerebellar molecular layer insinuating between the neural structure. The smooth surface Bergmann glial cell cytoplasm can be seen surrounding longitudinal or cross fractured parallel fiber bundles, Purkinje dendritic branchlets and stellate and basket cell axons. The Bergmann

glial processes encapsulate the spine synapses of granule cell axons and climbing fibers, surrounding the pre- and postsynaptic structures.

Reviewer I: Do all presynaptic IMPs represent Ca^{++} channels?

Authors: The large intramembrane particles observed in the P face presynaptic membrane seem to be permanent specializations involved in vesicle discharge. Their function is not yet known, but they may represent the Ca^{++} channels. This idea would be consistent with the voltage clamp data obtained by Llinás (Llinás RR. 1982, Calcium in synaptic transmission. Sc. Am. 247(4), 56-65) which indicate that the synaptic delay between the onset of the Ca^{++} current and the release of the transmitter at the squid giant synapse is short. This short latency suggests that the Ca^{++} channels and the vesicle release sites are very near to one another (Gershon MD; Schwartz JH, Kandel ER. Morphology of Chemical Synapses and Pattern of Interconnection. In: Principles of Neural Science. Kandel ER, Schwartz JH (eds). Elsevier Science Pub. Co. New York, 1985, pp 133-147).

Two decades ago, Katz and Miledi proposed that Ca^{++}, brought into the terminal by the action potential, interacts with specialized release sites inside the terminal, where it causes or facilitates a transient fusion of the vesicular membrane with the terminal membrane, thereby enhancing the probability that a given quantum of transmitter will be released (Katz B; Miledi R, 1967. A study of synaptic transmission in the absence of nerve impulse. J. Physiol. (Lond.) 192, 407-436). The fact that Ca^{++} acts so rapidly once its channels are open suggests that the Ca^{++} channels must be located near the active sites where the vesicles fuse to the membrane (Kandell ER. Factors controlling transmitter release. In: Principles of Neural Science. pp 120-131, above cited).

According to Kandell, one possibility is that Ca^{++} simply acts to facilitate directly the physical fusion of two lipid bilayer membranes, that of the vesicle membrane and that of the external membrane. A second possibility is that Ca^{++} acts through one or more Ca^{++} -sensitive proteins such as calmodulin, a calmodulin-sensitive protein kinase, or a phospholipid kinase to accomplish vesicle fusion.

Other presynaptic IMPs may be structural proteins to which synaptic vesicles attach during the exocytotic process. Some IMPs could also be related with endocytotic events associated to recycling of vesicle membranes.

Reviewer I: The designation of IMPs on postsynaptic processes as glutamate or aspartate-receptors. Certain of the IMPs may subserve those two functions, but it is clear that most IMPs subserve other functions than those listed.

Authors: In the present study some IMPs have been tentatively correlated with glutamate or aspartate receptors. This is just a speculation based on recent biochemical studies. Current ideas about postsynaptic membrane mechanisms suggest that the receptor complex mediates two functions: 1) binding of the transmitter (receptor function) and 2) forming a channel in the membrane through which ion flows (ionophore). All ion channels are large integral membrane protein complexes which form a tubular structure that transverses the lipid bilayer. Some IMPs might correspond to Na^+, K^+ or Cl^- channels. But not all receptors directly mediate changes in ionic conductances. An important class of receptors responds to the binding of the chemical transmitter by changing the metabolic machinery of the postsynaptic cell through a mechanism involving the formation of intracellular second messenger, cyclic AMP or certain lipids. In this context, some IMPs might correspond to the transducer or coupling protein associated to the catalytic subunit of adenylate cyclase, which catalyze the conversion of ATP to cyclic AMP. The G protein is not an integral membrane protein, but only a loosely associated peripheral membrane protein.