A REVIEW

Ultrastructural Details of some Invertebrate Peripheral and Central Nerves as Revealed by thin Sections and Freeze-Fracture Replicas

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INTRODUCTION

A neuron is a complex cell with many processes coming out of its perikaryon. These processes are the numerous dendrites and a single axon. The dendrites of different thickness branch in all directions, dividing and subdividing to form a complex arborescence. Besides, processes from other neurons end up either on the perikaryon surface, or on the dendrite surfaces. Moreover, by adding to all this the glial satellite cells, whose processes infiltrate all the empty spaces left among the neural branches, we come to a very complicated set-up, which is known as neuropile. The cell bodies or perikarya of both neurons and glial cells are embedded into this sponge-like mass. This is the basic structural organization at the level of the central nervous system (CNS) (1).

Out of all the neuron processes, there is only one, the axon, which is centrifugally bound and extends directly to its terminal with none or a few branches. Its length is determined by theesituation of the target to which the axon is aimed, and along its parthway, glial satellite cells are sequentially arranged around the axon surface from its emergence at the axon hillock to its final target ending. The nerve fiber then is the result of this sort of symbiosis: the central core formed by the axon and the external cover formed by the glial satellite cells. As known, in the peripheral nerve, the glial satellite cells are the Schwann cells (1).

The nerve fiber is considered to be the anatomical and physiological unit of the nerves. However, it is a peculiar unit, since it is formed not by one cell, but by a combination of two cell types closely apposed and physiologically coupled, though independent.

A variable number of nerve fibers grouped together form a fascicle. In the fascicle, loose connective tissue (fibrocytes and fibrils) is interposed among the nerve fibers. Each fascicle

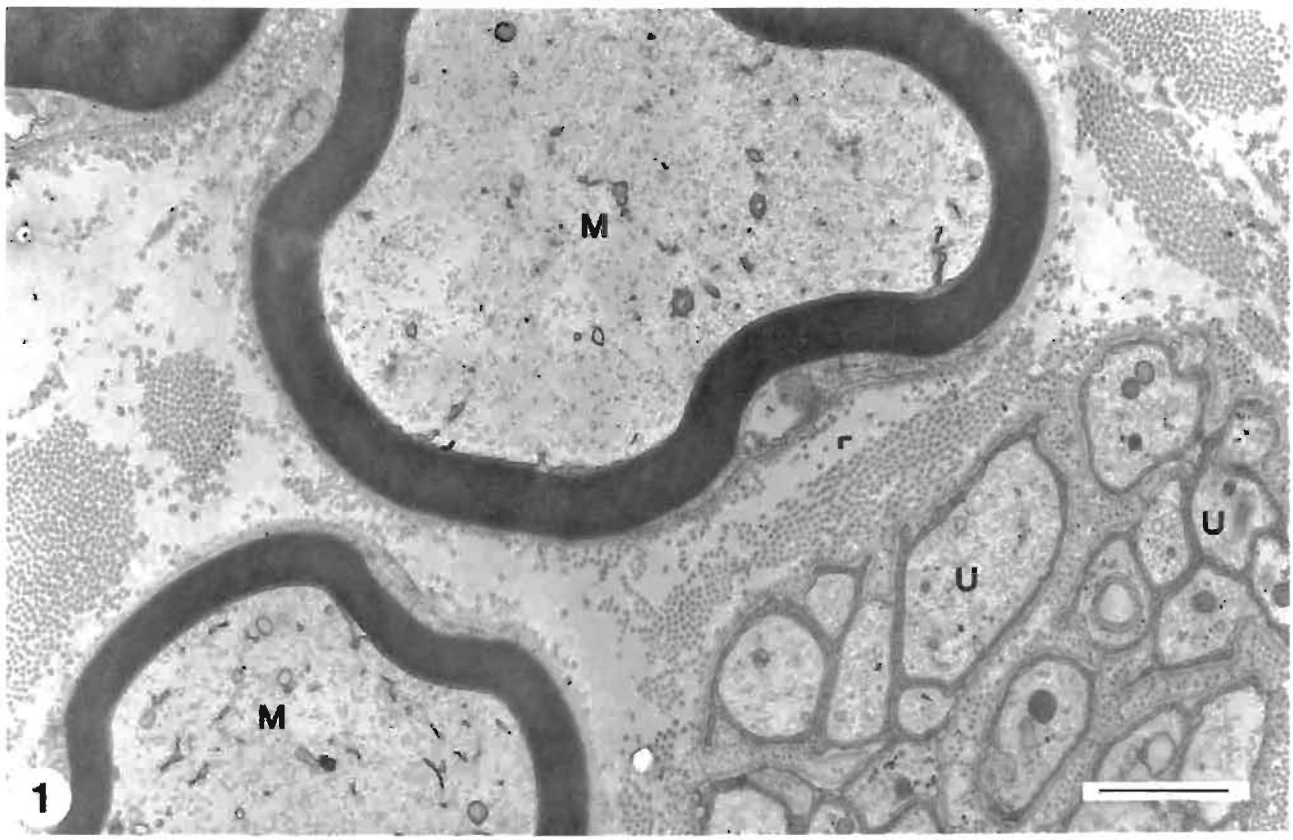


Fig. 1 Cross section of a rat sciatic nerve exhibiting the two types of nerve fibers integrating the vertebrate peripheral nerve: myelinated (M) and unmyelinated (U) ones. Scale - 1 μm.

is individualized by a peripheral wrapping know as the perineurium, the cells constituting it being attached by tight junctions (2). The perineurium behaves as a barrier to diffusion similar to the blood-brain barrier of the central nervous system (3-5), opening the perineurium being necessary in order to see the penetration of electron-dense tracers (6) such as thorium dioxide, lanthanum, ferritin, etc.

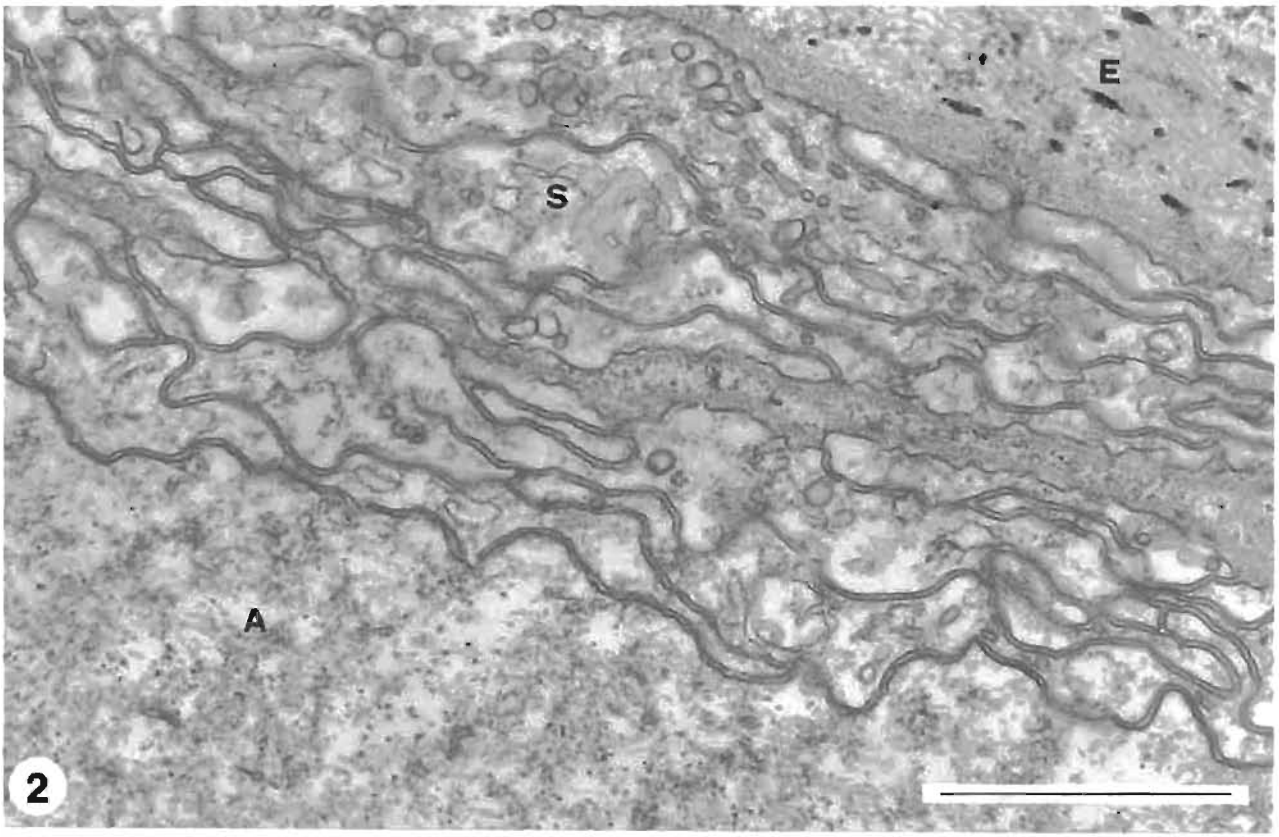
The Vertebrate Peripheral Nerve Fibers

In the vertebrate, two types of nerve fibers, the myelinated and the unmyelinated ones, coexist in the fascicle, the differences being, the myelin sheath interposed between the axon and its satellite Schwann cell in the myelinated fibers, and the number of axons related to a single Schwann cell in the unmyelinated ones (Fig. 1).

Fig. 2 Cross section fo the squid *Dosidicus gigas* giant nerve fiber showing part of the axon (A), the Schwann layer (S) with the extense and elaborated interdigitations of the Schwann cells, and the endoneurium (E). Scale = 1 μm.

Fig. 3 Electron micrograph of a squid *Spioteuthis sepioidea* nerve fiber showing the diffusion of thorium dioxide from the endoneurium (E) to the axon (A) surface. The electron-dense marker penetrates the intercellular clefts of the Schwann cell layer (S) to reach the axon-Schwann cell interspace. Scale = 0.5 μm.

Fig. 4 Axon (A)-Schwann (S) cell boundary of a giant nerve fiber of squid *S. sepioidea*. A structural complex (arrow) involves both the axon and the Schwann cell plasma membranes. Note the characteristics of the complex: the subaxolemmal density, the conspicuous trilaminar substructure of the axolemma and the close apposition of this latter membrane to the adjacent Schwann cell membrane. Scale = 0.1 μm.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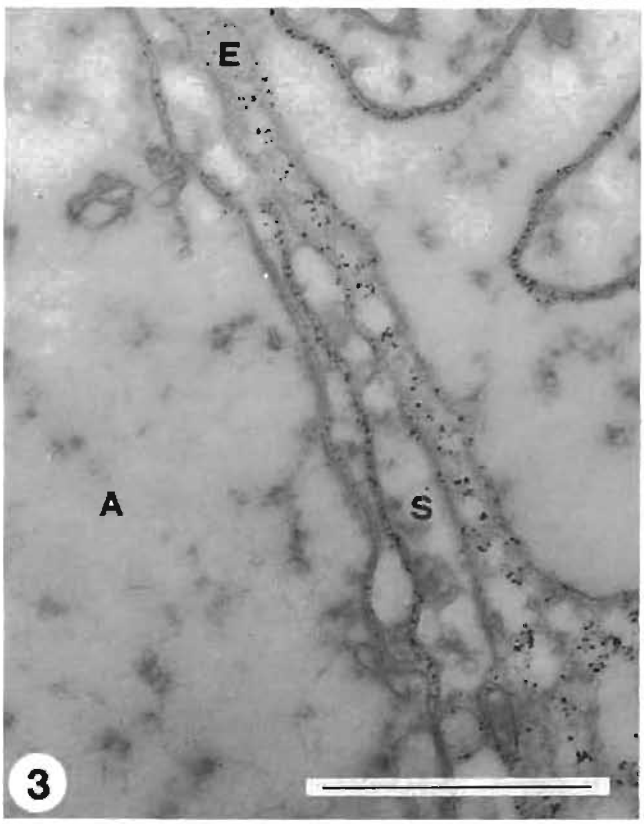
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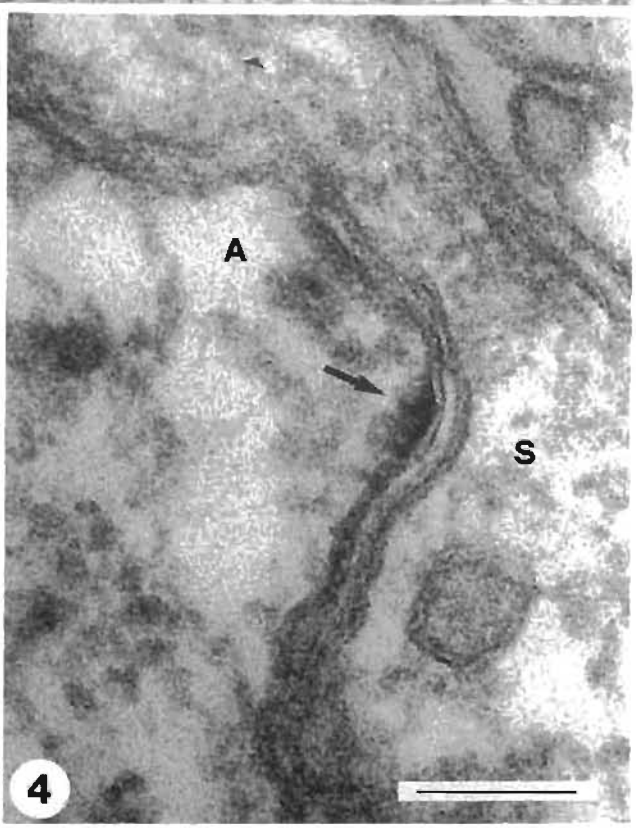
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The myelin sheath, as demonstrated by Geren (7), is the plasma membrane of the Schwann cell spirally wrapped around the axon. A mesaxon is first formed by the closed apposition of the two lids of the Schwann cytoplasm, once the axon has been completely surrounded by the satellite cell. Then, the mesaxon elongates and wraps itself as a spiral line. The cytoplasm of the Schwann cell is interposed between contiguous turns and later is squeezed out, myelin becoming a compact multilaminated structure.

If the myelin sheath is a dependency of the Schwann cell plsama membrane, and the Schwann cells are individuals lined around the axon, then, at each cell ending, the myelin sheat also comes to an end, and forms the structure known as node of Ranvier. Since myelin is formed by the spiral wrapping of a shovel-like sheet (8), its longitunidal section at the level of a node characteristically appears as double dense lines obliquely directed towards the axon surface and arranged one after the other, with Schwann cell cytoplasm interposed between two neighboring ones (9,10). The outermost double line segment reflects itself and is continuous with the Schwann cell plasma membrane. At this site, this membrane forms finger-like processes which interdigitate with the ones belonging to the adjacent Schwann cell. In such a way, the axon below is protected by a loose layer which allows a free and rapid diffusion. This arrangement seems to be the morphologic correlation for the saltatory conduction (11).

Webster et al. (12) demonstrated that there is a sequence in the myelinating process. In the embryonary state, all the axons are unmyelinated. There is a sort of recognition of certain axons by the neighboring Schwann cells, and those axons intented to be myelinated, are first segregated towards the periphery of the bundle, then are segregated by a sort of tongue from the encircling Schwann cell, and later isolated inside a pocket of this satellite cell. Only when this one-to-one axon-glia relationship is attained, myelination occurs.

Invertebrate Peripheral Nerve Fibers

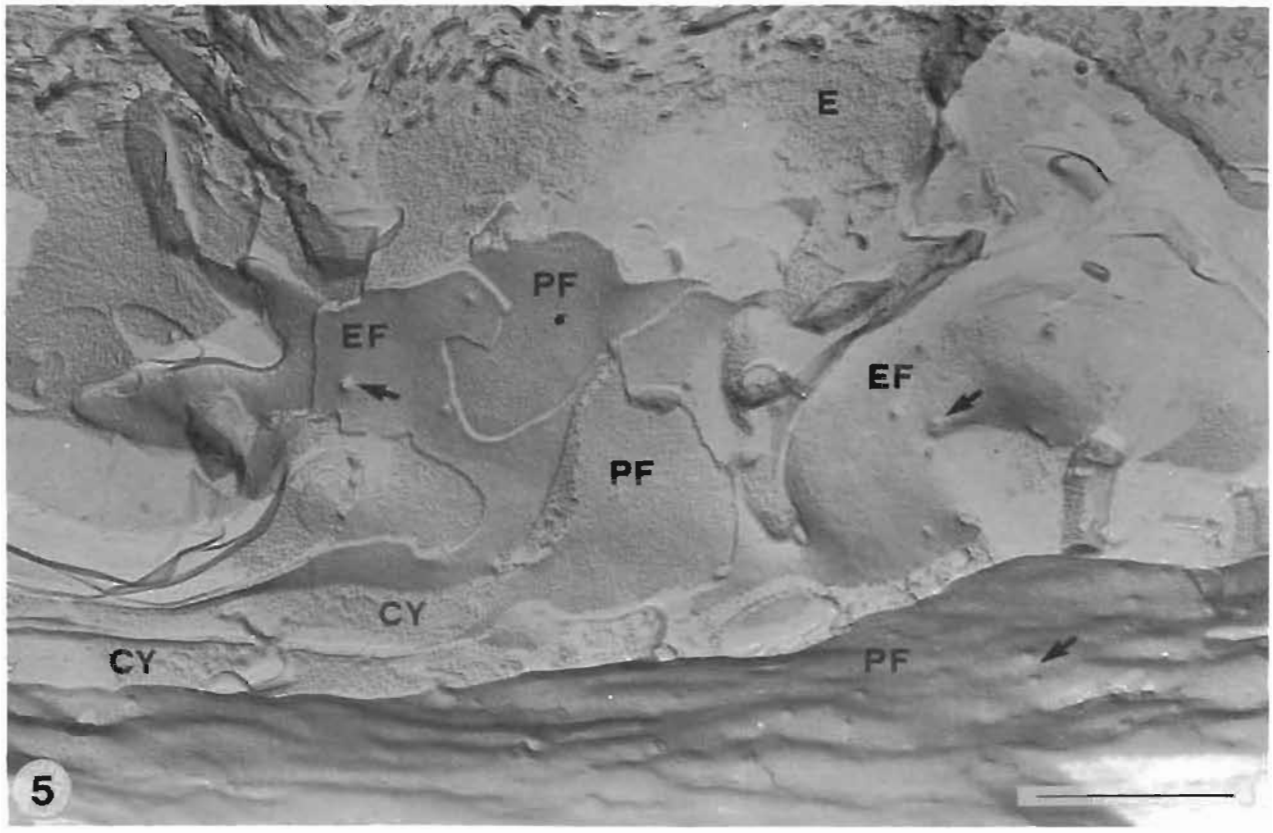
Other types of axon-glia relationship, according to the axon diameter and the number of axons occurring in the same single fiber in the invertebrates are found. Thus, in the squid and also in lobster, crayfish and polychaetes, wich posses only unmyelinated fibers, different types of axon-Schwann cell relationship have been found: the ones existing in the vertebrate peripheral and central nervous systems and also a special one originated by the presence of giant axons in those animals (13). In vertebrates, the rapid nerve conduction is accomplished by insulating the nerve with the myelin sheath; in the case of the above-mentioned invertebrates the fast conduction is achieved by increasing the axon diameter, thus establishing a new axon-glia relationship. In order to encircle the large axon perimeter, it is necessary to multiply the number of Schwann cells in a cross section. These cells are closely apposed to the axon surface, and their ends are highly interdigitated (Fig. 2), thus creating tortuous intercellular pathways that connect the periaxonal space with the bulk of the extracellular space represented by the endoneurium (14,15).

In the squid, each Schwann cell is a rectangular sheet measuring about 45 by 60 μm., with a variable thickness ranging from 0.2 μm in some species (16), up to 6 μm in the giant squid *Dosidicus gigas* (17).

The permeability of the intercellular spaces of the Schwann cell layer was presumed from early studies on the diffusion of K^+ (18) and tritiated water (19) and later shown by us by using thorium dioxide as a marker (Fig. 3) (20). In such a way, it was demonstrated that the only continuous barrier interposed between the axon cytoplasm and the bulk of the extracellular space was the axon plasma membrane or axolemma.

Fig. 5 Freeze-fracture replica of the squid giant nerve fiber showing several membrane faces (EF and PF) in the Schwann layer corresponding to fractured interdigitations. Note the protuberances on the E-face and the corresponding pits on the P-face (arrows). CY, Schwann cell cytoplasm and E, endoneurium with fractured collagen fibrils. Scale = 1 μm.

Fig. 6 and 7. Replicas exhibiting the axon (A)-Schwann cell (S) boundary in squid giant nerve fibers. Note the ridges (arrows) formed by IMPs linearly arranged on the P-face of the axolemma and on the E-face of the Schwann cell adaxonal membrane. This arrangement could be related to the sites of axo-glial coupling represented by the structural complexes. E, endoneurium. Scale = 1 μm.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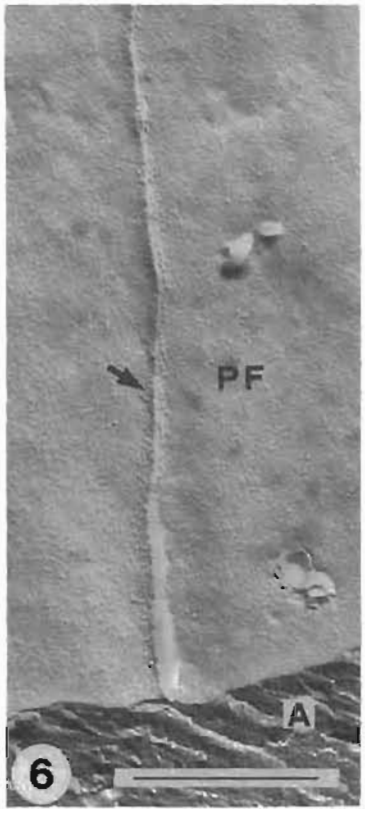
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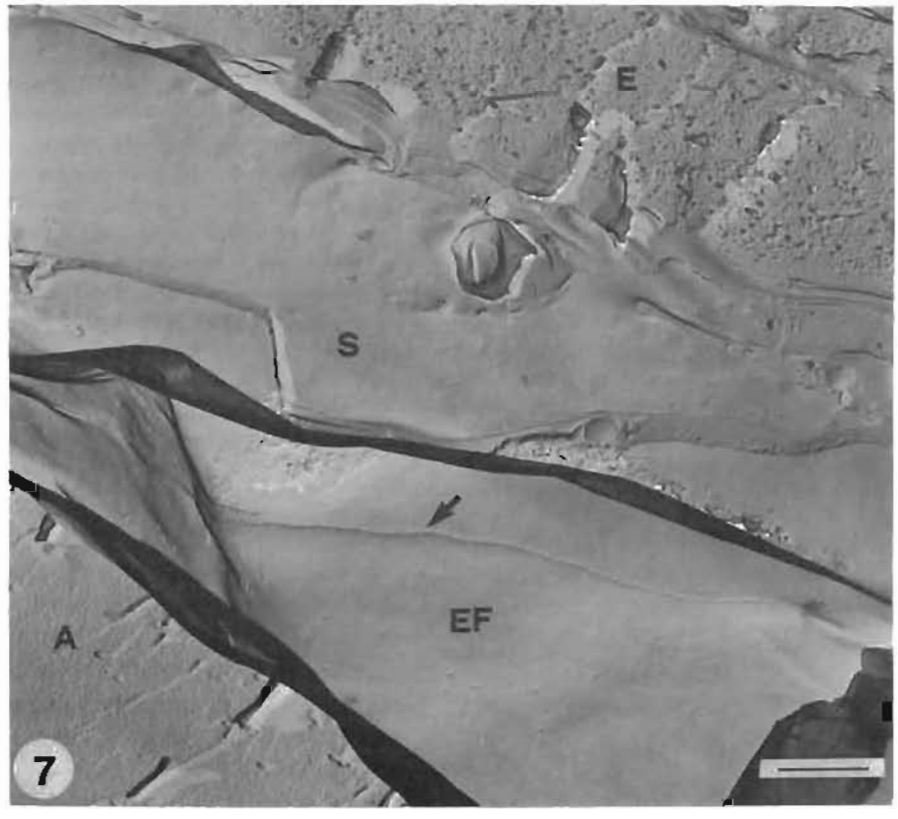
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This membrane can discriminate by size ions and molecules, and therefore it was pointed out as the excitable membrane of nerve (21).

As any other cell plasma membrane, the axolemma appears in the electron microscope as a trilaminar image, 80 to 100 Å thick, its thickness increasing with the aging of the animal up to a plateau of about 105 Å (13). Structural complexes irregularly spaced along the axon-Schwann cell boundary have been described (22). They are characterized by three mainly morphologic features: (a) the constant trilaminar substructure which confers a sort of rigidity to the axolemma at such a level; (b) the existence of an electron-dense material lining this membrane on its axoplasmic aspect, similar to the post-synaptic dense material; and (c) the tapering off of the axon-Schwann cell interspace to total disappearance by the close appostion of both plasma membranes (Fig. 4). These complexes seem to be specialized zones for active transport and also sites of coupling of the axon and its satellite glial cell mediated by a cholinergic system (23).

Recent studies using the freeze-fracture technique (24) have confirmed the arrangement of the various cel layers in the giant fiber of the squid, but also have revealed some other features of the membrane faces inaccessible to thin sections. Protuberances and their complementary pit images have been observed in the axolemma fractures faces, as well as in the neighboring Schwann cell membrane (Fig. 5). These images could be the morphologic correlates for the exchange of substances, be acetylcholine, proteins or some other macromolecules, between the axon and its satellite glial cell, as demonstrated by Lasek and Tytell (25) and by Inoue et al. (26).

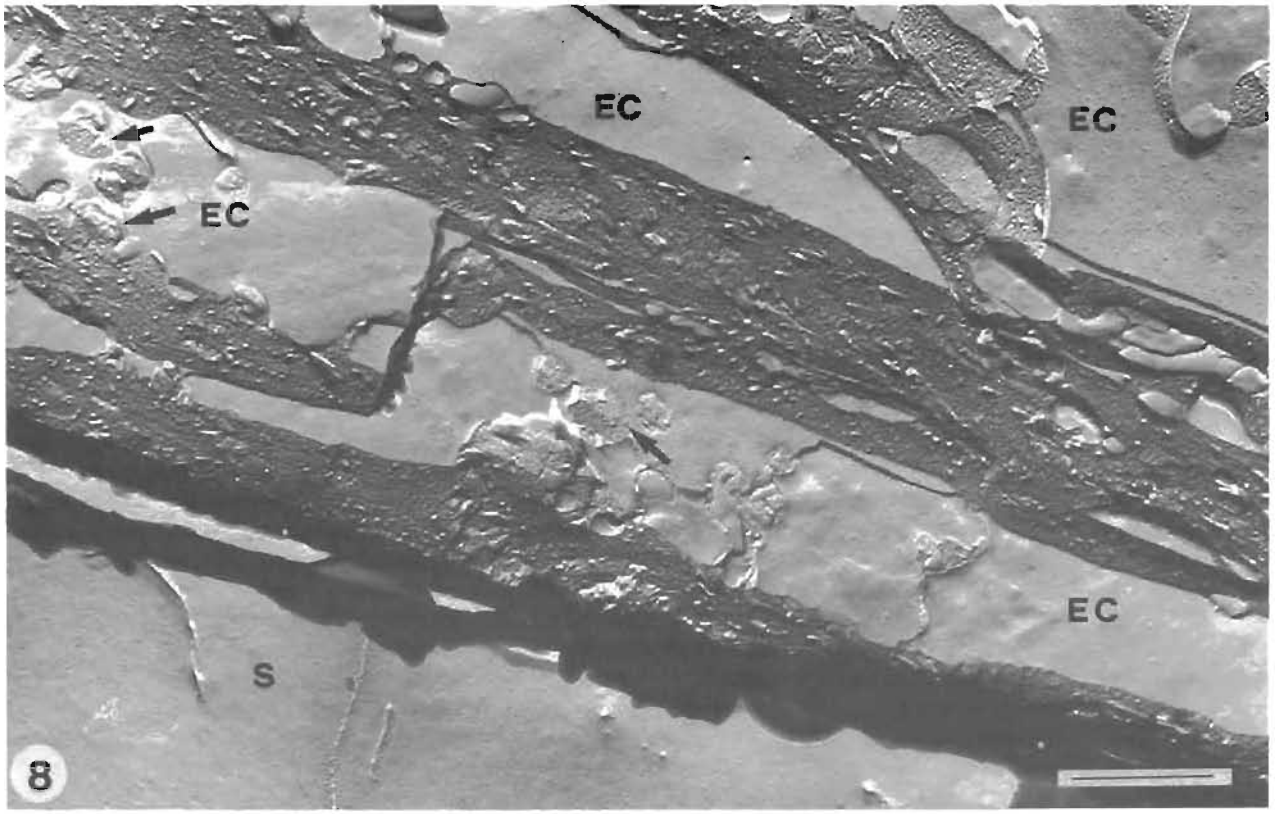
Besides, presence of intramembranous particles (IMP) in longitudinal arrangements or forming ridges, in both the axolemmal face and the adaxonal Schwann cell membrane face (Figs. 6 and 7) may be correlated with the sites of axo-glial coupling, corresponding to the structural complexes observed in thin sections. the axolemma P-face possess numerous small IMPs and also larger ones, 10-16 nm, similar to those suggested to represent some sodium channel components in the squid *Loligo* (27).

The smaller IMPs, more numerous, are not uniformly scattered in the P-face; on the contrary they tend to cluster in zones separated by areas having no or only a few particles. This arrangement was first reported by Chang & Tasaki (27) and more recently observed by us in the giant axon of the lobster (28).

At the axon periphery, most of the cytoplasmic components seem to be more concentrated (29) (Figs. 2 and 7). Vesicular profiles situated very close to the surface have been described by Hodge & Adelman (30) as radially oriented cisterns of the agranular reticulum. In addition, Henkart et al. (31) have pointed out the close relationship of these peripheral cisterns and the sequestering of calcium ions penetrating to the axoplasm during stimulation. In replicas, these subaxolemmal cisterns have been confirmed in the squid (24), whereas, in lobster giant axons the most numerous and conspicuous organelles subjacent to the axolemma are mitochondria (28). In the case of the squid axon, the cytoskeleton, and specially the microtubules contributing to its formation, have been pointed out as structures involved in the functioning of excitable membranes, its probable function being to control the spatial interaction of proteins regulating the ionic permeability. This is the hypothesis proposed by Endo, Sakai & Matsumoto (32), and by Metuzals and Tasaki (29). Also, the coiling and uncoiling of the filaments, as well as their sliding in the network could also account for the mechanism of axonal transport (33).

Beyond the adaxonal glia or Schwann cell, the giant nerve fibers are individually ensheathed by a special endoneurial arrangement formed by layers of cells interleaved with collagen-filled spaces. Endoneurial cells exhibited different features in replicas of different invertebrate species. In the squid (Fig. 8), replicas show abundant cross-fractured infoldings in the cell membrane, some of them continuous with the

Fig. 8 Fracture plane through the endoneurium depicting the endoneurial cell (EC) interleaved by spaces containing collagen and extracellular matrix (dark, wide spaces). Intracellular invaginations (arrows) of the matrix material are observed. S, Schwann cell adaxonal membrane. Scale = 1 μm.
Fig. 9 and 10. Freeze-fracture replicas of the endoneurium of the giant nerve fiber of lobster. As observed, the endoneurial cells exhibit large amounts of exo-endocytic *profiles with protuberances* in the E-face and their complementary *pit images* in the P-face. Scale = 1 μm.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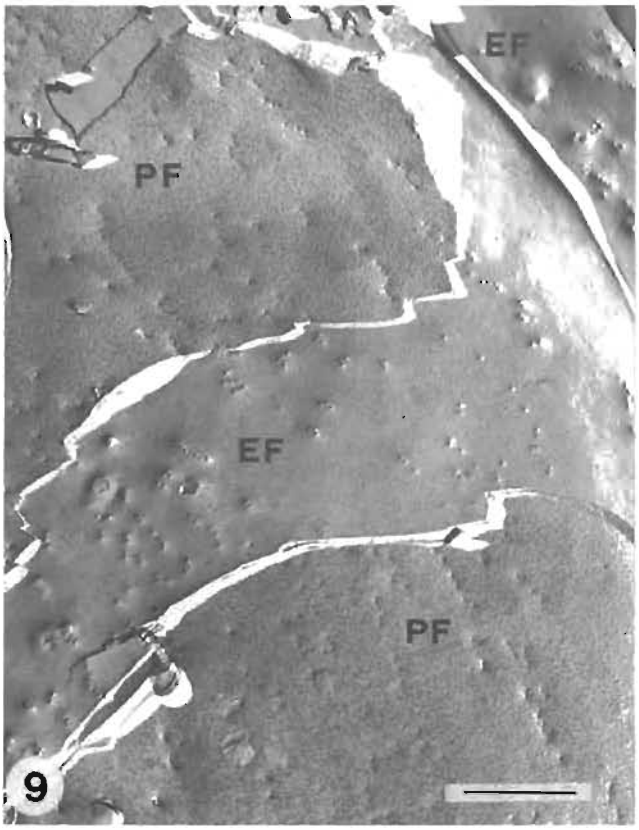
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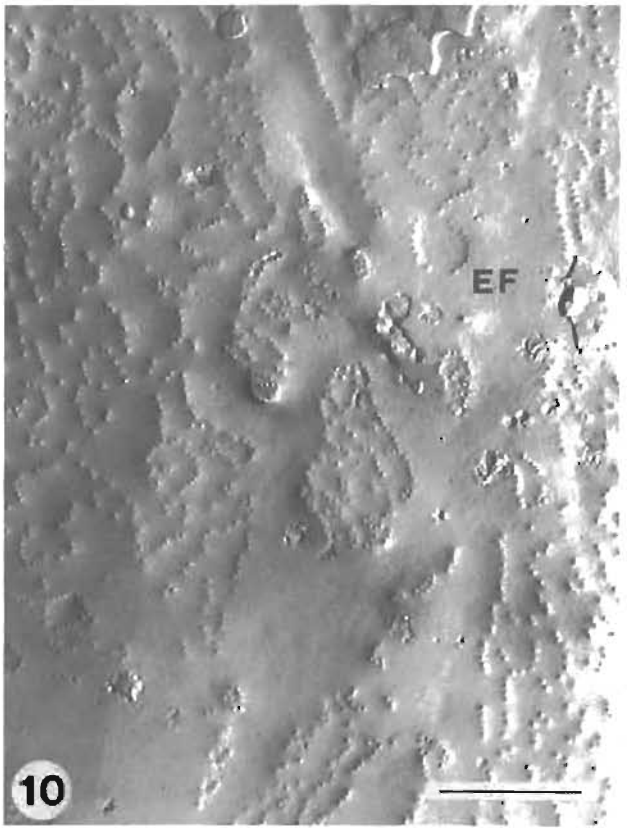
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matrix of the collagen-filled spaces, others look-like fractures through cytoplasmic interdigitations between adjacent cells (24).

In the lobster (28), the image of the fractured endoneurial cells is dominated by an exceeding number of exo-endocytic profiles (Fig. 9 and 10). Besides, clustered IMP images corresponding to intercellular gap junctions, as well as linear arrangement of IMPs forming incomplete, facia type, tight, junctions are observed.

Finally, in the crayfish (34), fracture replicas reveal conventional features in the endoneurial sheat cells, as in any other cross-fractured cell membrane (IMPs, exo-endocytic profiles) and also special images of large, grouped craters which have been associated to the openings of the cytoplasmic tubular lattice system. This system, first reported in the Schwann cells of the lobster giant nerve fibers (35) has been occasionally observed in the same cells of the squid giant fiber (16). However, in the crayfish, lattices are numerous in the adaxonal glia, as well as in all the cell layers conforming the endoneurial sheath (36). The tubular lattice has been postulated as a facilitated transcellular pathway for ions and molecules accounting for the unrestricted permeability noted in crayfish nerves. In the lobster, however, more recent studies (28) have shown that the tubular lattices, though present, play a lesser role in the movement of solutes than in the crayfish nerves. In the lobster, the outer sheath or epineurium associated to an endoneurial ensheathment of greater thickness is more effective in hindering diffusion of ion and molecules towards and from the axon surface.

Central Nerve Fibers of Vertebrates and Invertebrates

The squid optic nerve, as its homologous the olfactory nerve of the garfish (37), are central nerves formed by unmyelinated nerve fibers. Thousands of small axons are packed together in fascicles, each fascicle being individualized by a peripheral glial ensheathment. Axons are apposed one to another without any intervening cell process; therefore, axolemmas of adjacent axons are close together, with only a thin intercellular space separating them. A few trabeculae coming from the peripheral ensheathment penetrate the bundle of axons and separate large groups of them inside the fascicle.

Such organization makes these nerves interesting preparations for biochemical and biophysical studies on the excitable membrane. In fact, the favorable axon-glial cell membrane relationship has made it possible to obtain from those nerves membrane fractions enriched 4-5 fold in axon excitable membrane (38,39), as compared with similar fractions obtained from squid (40) and lobster (41) peripheral nerves.

Fine structure details of optic nerves in vertebrates have been widely reported (42-45), specially those concerning the organization of the myelin sheath. On the other hand, only brief descriptions of the same nerve in squid have been included in some papers (39,46).

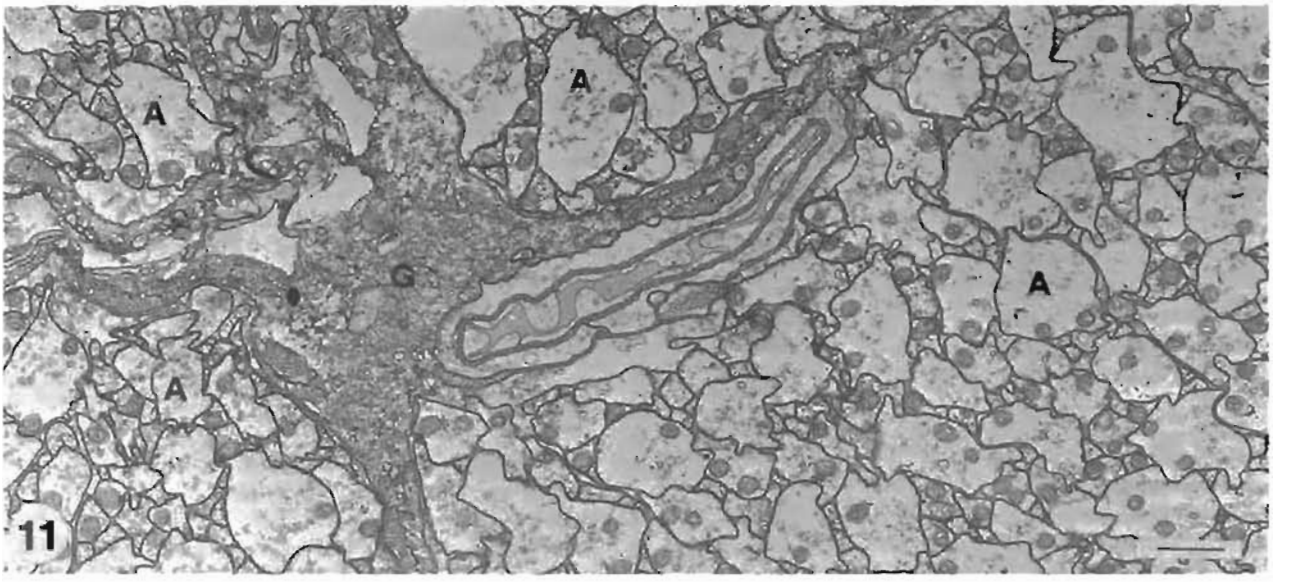
When observed in the electron microscope, each fascicle of the optic nerve of the squid *Sepioteuthis sepioidea* is made up of thousands of axons, 130 nm to 2 μm in diameter each (Fig. 11). An ensheathment formed by at least two layers of glial cells separated by spaces filled with a fine granular matrix surrounds the fascicle on the outside. Collagen fibrils and blood vessels are observed embedded in that matrix, as well as in the trabecualae penetrating from the ensheathment.

Axons exhibit as the most conspicuous organelles, abundant mitochondria, sometimes

Fig. 11 Thin section of the optic nerve of squid *S. sepioidea* showing part of bundles of axons (A) separated by trabeculae of the glial ensheathment (G). Axons of differents diameters are yuxtaposed without intervening glial cytoplasm. Note the large amount of mitochondria in the axons. Scale = 1 μm.

Fig. 12 Freeze fracture replica of the squid optic nerve. Axons, lying in the same direction, exhibit different diameters along their trajects and spherical profiles (mostly mitochondria, arrows) in the cytoplasm. Note the serrated fracture border of the E-face (EF). PF, P-face. Scale = 0.5 μm.

Fig. 13 A high-power view of a replica of the squid optic nerve showing the irregular distribution of the IMPs on the axolemma P-face (PF). Two main types of particles according to their diameters are observed in this membrane face. Some clusters of small particles resemble incipient gap junctions (arrows). Scale = 0.5 μm.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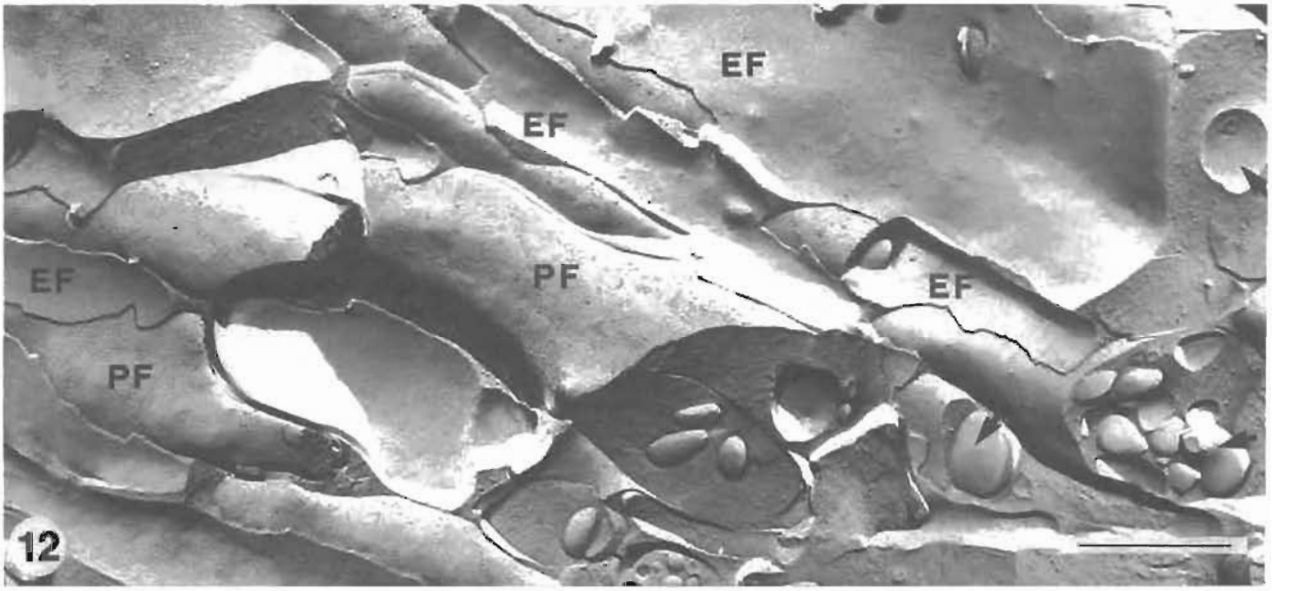
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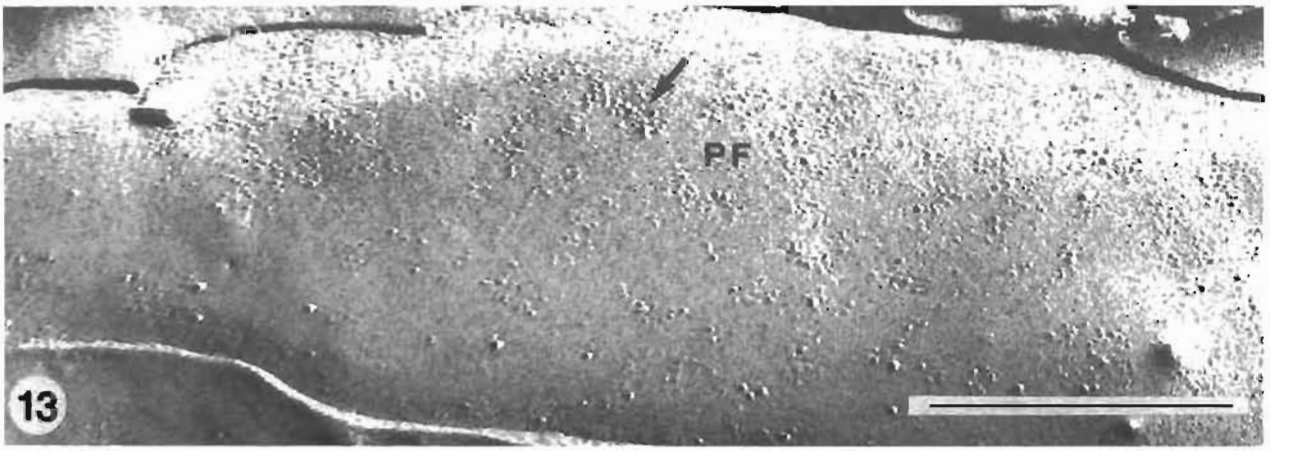
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being as large a to occupy a large portion of the axoplasm cross section.

Freeze-fractured replicas show sequences of mostly longitudinal, fracture membrane faces corresponding to alternated axolemmal P and E faces (Fig. 12). In some instances membrane faces of glial cells and blood vessels integrating the trabeculae are also seen. The axolemma E-face appears to fracture along serrated borders and exo-endocytic profiles protruding from sharp peaks of those borders are observed (Fig. 12). The P-face shows, as usual, a high concentration of IMPs. In this case the particles are concentrated in areas separated by zones devoid of particles. This distribution of IMPs has also been reported in the axolemmas of the squid (24) and lobster (28) peripheral giant axons.

In the areas of high particle concentration, clusters of tightly-packed IMPs resembling incipient gap junctions are also observed (Fig. 13). Exo-endocytic profiles are commonly seen in both faces, being the protrusions on the E-face and the complementary pits on the P-face.

Glial cell membranes appear as extended and irregularly-shaped fractured faces with IMPs of different sizes regularly scattered. Fractured, small processes and exo-endocytic profiles are also apparent.

CONCLUSIONS

The present work is a review of the morphological organization of some invertebrate peripheral and central nerves, as revealed by the electron microscope. It has also been tried to correlate the ultrastructural details with some functional and biochemical findings reported in those nerves.

The ultrastructure of vertebrate peripheral nerves has been briefly dealt with in order to compare the axon-glia relationship, exhibited by such nerves, with that occurring in the invertebrate ones. In these latter nerves, axons with the largest diameter (giant axons), as well as axons with the smallest diameter occur, thus determining peculiar morphologic organization of the axon and their surrounding satellite glial cells.

Giant axons, as well as giant neuronal cell bodies, have been for almost half a century ideal preparations in the neuroscience research field. A great body of knowledge has been accumulated, and continues to accumulate, after the existence of giant nerve fibers and giant neuronal bodies in invertebrates were pointed out to the neuroscientists. The use of these giant cells, though mostly of mollucan origin, has played a leading role in the elucidation of the mechanisms of nervous conduction. The role of ions and molecules, the finding of the ionic channels in the membrane, membrane biochemistry and neuropharmacology, are some of the knowledges achieved with the use of these preparations.

On the other hand, the study of the morphology of the central nerves, optic of the squid (38) and olfactory of garfish (37), has contributed specifically to membrane biocehmistry. The molecular composition and structure of the axon membrane must be known in order to achieve a better understanding of the mechanisms of excitation in nerve. The identification of the axon excitable membrane (21) was followed by attempts to isolate it and obtain membrane preparations enriched in axolemma and having a good yield. This was first accomplished with stellar nerves of giant squid *D gigas* (40) and later with the optic nerves of the same species (38). It was then pointed out the importance of the morphological organization of these latter nerves which led to the obtention of an axolemma-enriched membrane fraction in larger amounts than the ones obtained from the stellar nerves. The same morphologic criterion was applied to favor the use of the olfactory nerve of the garfish (37) and of the lobster walking leg nerves (47,48) for the obtention of isolated excitable membrane preparations. Furthermore, the solubilization of the sodium channel protein (49,50) whic led afterwards to the cloning of its cDNA (51) were achieved by the use of membrane preparations. The same can be said, as well, with respect to the studies on characterization of single ionic channels incorporated in lipid bilayers (52).

Finally, it is expected that the structural details revealed by freeze-fracturing of the squid optic nerve membranes, as reported in the present paper, will be useful for the understanding of some physiological facts occurring in those nerves in which naked axons are closely-packed, without any intervening glial cell cytoplasm separating their adjacent excitable membranes.

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RESUMEN

El presente trabajo es una revisión de la organización morfológica de nervios periféricos y centrales de algunos invertebrados a nivel de microscopía electrónica. Se correlacionaron además, los detalles ultraestructurales con hallazgos funcionales y bioquímicos reportados en los mismos nervios.

La existencia de axones de gran diámetro (gigantes) determina relaciones morfológicas peculiares del axón y sus glias satélites cuando se las compara con la relación axón-glia que ocurre en los nervios periféricos de los vertebrados.

Los axones gigantes y las neuronas gigantes han jugado papel fundamental para la elucidación de los mecanismos de la conducción nerviosa: el papel de iones y moléculas, y el hallazgo de los canales iónicos de la membrana celular. Además, la bioquímica de la membrana y la neurofarmacología son también conocimientos logrados con estas preparaciones.

La identificación de la membrana excitable (21) fue seguida por intentos de aislarla, lo cual se logró primero con nervios esteleares de calamar *D. gigas* (4Ø) y después con ópticos de la misma especie (38). Se hace hincapié en la importancia de la organización morfológica de los nervios centrales, óptico de calamar y olfatorio de pez gar (37), que por estar constituidos por numerosos axones amielínicos, muy pequeños, exhiben una relación glia-neurona exactamente opuesta a la de las fibras gigantes, lo cual favorece la obtención de fracciones enriquecidas en membrana excitable. Los nervios periféricos de langosta marina ocupan una posición intermedia en cuanto a este rendimiento bioquímico, pero todavía favorable por el número de nervios que se obtienen por animal.

Esta revisión incluye además, detalles ultraestructurales de nervios ópticos de calamar revelados por la técnica de criofractura. Se espera que ellos sean relevantes para la compresión de hechos fisiológicos que suceden en estos nervios, donde axones desnudos yacen cercanamente empaquetados sin que extensiones gliales separen sus membranas excitable adyacentes.

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