The neuroglia of the rat optic nerve. Part I. Golgi-Hortega and Golgi-EM studies

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SUMMARY

In the present study, one describes the morphology of the macroglia (astrocytes and oligodendrocytes) of the optic nerve of the albino rat, having been stained according to the modification of del Rio-Hortega of Golgi-Kopsch's method. This cytology has been studied in the intra-orbital optic nerve, in the intracranial portion of the same, in the optic chiasm and in a first segment of the optic tract.

Astrocytes show small differences between those located in the marginal zone of the nerve and those located in the center, being all of them classified as belonging to the fibrous type of astrocytes. With their extensions, these cells establish contact with the external limiting membrane, blood vessels and myelinic nerve fibers. On the level of the limiting membrane, their terminal feet form a kind of cellular barrier similar to that of the cerebral cortex in contact with the pia mater. With the nervous fibers, they establish close relationships at Ranvier's nodes level, being arranged around them forming small rings.

The oligodendrocytes show their main branches impregnated up to where they reach the myelin sheath, but the protoplasmic compartment of this one is not impregnated, giving a different image to the one seen after microinjections with markers.

Morphometric studies using fractal dimension and related parameters are presented next in part II of this work.

Keywords: Optic nerve – Astrocytes – Neuroglia – Golgi-method – Golgi-EM

INTRODUCTION

The main virtue of the method described by Golgi (1873) is to reveal the complete shape, the silhouette, of nerve and glia cells of the central nervous system. The number of cells that may be impregnated according the "*reazzione nera*" is very small, around 5% of them, which, far from being a disadvantage, allows the visualization of the cells stained in their entirety without important superposition of cellular structures.

The performance of this method has been improved by the introduction of numerous variants and modifications, of which the Cajal ones should be highlighted, which made it much more productive and ultimately reliable (Cajal and de Castro, 1933; Valverde, 1965).

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However, impregnation of neuroglia cells with the Golgi method is somewhat more difficult, and that is the reason why variants have been devised in which different chemical components have been introduced, such as mercury salts (Cox, 1891), formalin (Kopsch, 1896), and, in more recent times, glutaraldehyde (Colonnier, 1964; Braitenberg et al., 1967).

Regarding neuroglia, undoubtedly an effective modification of the Golgi method was described by del Rio Hortega (1928) and collected in the methods book of Cajal and de Castro (1933), a variation in which, together with the potassium chromium salt and formaldehyde, the chloral hydrate interacts with the tissue, being the technique used by us in this study.

In order to analyze the relationships of the astrocyte prolongations with the neighboring nerve fibers, as well as with the superficial limiting membrane of the nerve, the one which is in contact with the meninges involving the optic nerve, the gold-toning technique for electron microscopy was applied to selected sections stained according the Golgi-Hortega method, an approach not used till now for neuroglia cells (Fairen et al.,1977; Mestres and Schneider, 1997).

In a second part of this paper, the cell shape of the astrocytes of the optic nerve has been investigated, determining their fractal characteristics and related parameters.

MATERIAL AND METHODS

Golgi method. Light microscopy

In the 1980s, series of rat brains treated according to the Golgi-Cajal method (Valverde, 1965), as well as the Cox method (1891) and Hortega's modification of Kopsch's method (1828), were prepared in our laboratory in Homburg Saar and stored in our institute archive, which has been consulted for the present investigation.

The mentioned collections were prepared along several years (1984-1989) using approximately 150 adult Sprague-Dawley rats (200-230 g BW) of both sexes, which were sacrificed by decapitation with the aim to avoid the use of anesthetic agents (with permission of the Animal Welfare Commission of Saarland).

The brain was removed very quickly and, after washing in a buffered saline solution, they were segmented by cutting them in horizontal and coronal directions (slabs of 3-4 mm. thick) and immersed in the Rio-Hortega's solution (dichromate potassium 3 g, chloral hydrate 3 g, 10% formaldehyde 50 ml).

The solution became intensely cloudy within a few hours, so it was replaced by a fresh solution every 10-12 hours, working in total for about 60 hours at room temperature and protected from light. The tissue specimens were placed in a vessel with a porous porcelain basket hanging to prevent the tissue to come in contact with the precipitates of the Golgi-Hortega solution, which accumulated at the bottom of the vessel (Romeis, 1968). At the end of this step, the hardened pieces were washed in a 1.5% aqueous solution of silver nitrate, giving time to the solution to act on the surface of the tissue blocks, where soon silver chromate crystals appeared that were partly removed by brushing the surface gently. The tissue blocks were then transferred to a 1.5% silver nitrate aqueous solution, changing it every 24 hours and working for a total time of 3 days.

After impregnation, the blocks were embedded in celloidin following the protocol described by Ramon-Moliner (1970), and sectioned with a thickness of 50 to 70 microns in a sliding microtome (Jung, Heidelberg). The histological sections were dehydrated in an ascending series of alcohols, passed through xylol or toluol, and mounted on slides covered with Eukitt (Sigma-Aldrich) and a very thin coverslip, and examined and photographed in a light-microscope Vanox (Olympus).

The Golgi-Hortega protocol rendered surprising good results in the optic nerve, optic chiasma and optic tract, obtaining impregnations of astrocytes both in the periphery and in the central nerve area, as well as of the oligodendroglia cells (preliminary communication by Mestres, 1988).

Electron Microscopy - Golgi-EM method

Sections of rat brain and optic nerve stained according to the del Rio-Hortega method were

selected for further processing in electron microscopy. For this purpose, the gold-toning procedure was applied, which allows a considerable reduction in size of the silver chromate crystals, greatly facilitating a good ultra-structural examination of the sample (Fairén et al., 1977; Mestres and Schneider, 1997). After submitting the sections to gold-toning, they were post-fixed with osmium and dehydrated in an ascending series of alcohols, transferred anhydrous acetone or propylene oxide (Sigma-Aldrich) and infiltrated into Epon (Polyscience) and polymerized at 60ºC for 48 hours. Ultrathin sections of approximately 80 nm thick were obtained with a diamond knife (Diatome) on an ultramicrotome Leica-Ultracut S. The thin sections were stained with uranyl acetate and lead citrate, and examined and documented under a transmission electron microscope Zeiss EM CR.

RESULTS

In the three locations studied, intra-orbital optic nerve, optic chiasm and optic tract, a remarkable number of impregnated neuroglia cells was found. The range of impregnated cells observed was approximately 8% of the cells existing in each location (Cavalloti et al., 2000). These estimates were made taking as reference the average number of cell nuclei (234± 8) counted in cross sections of rat optic nerve with H&E stained (not shown).

Astrocytes

In the intra-orbital optic nerve, astrocytes in two different locations can be distinguished, some located in the marginal zone of the nerve close to the meningeal envelop, and others located in the central part of the nerve.

Marginal located astrocytes show processes that radiate towards the meningeal sheath, having others that go inwards and insinuate themselves between the nerve fibers of this nerve. In general, the prolongations of these so-called marginal astrocytes are quite robust and run in a transversal plane of the nerve, but some others are longitudinally oriented (Figs. 1, 2).

The central astrocytes give the impression that they have many more extensions, but these, unlike those of the marginal ones, are thinner and more delicate and in some cases are exhausted before they reach the surface of the nerve, however, many of them reach the limiting membrane (Figs. 1, 3). Relevant to typify the cell type is the fact that the processes and ramifications of these cells have a smooth surface, which allows us to classify them as fibrous astrocytes.

In the intracranial itinerary of the optic nerve, astrocytes maintain these morphological characteristics (Figs. 1, 2). In the chiasm and optic tract, the astrocytes become adapted to the cracks and slots between the nerve fiber bundles, and giving the impression that the extensions are arranged in planes determined by the nerve fascicles (Fig. 1).

For ultrastructural observations, the Golgi-EM method was used revealing the cell nucleus and the cytoplasm of the impregnated astrocytes, which present a thin layer of metallic deposits just below the plasma membrane. The cell nucleus shows a homogeneous distributed chromatin with only few condensations. The nuclear membrane is perfectly perceptible and, in the cytoplasm, there are few organelles, as well as gliofibril-bundles (Fig. 2). These structures, corresponding to the system of intermediary filaments, i.e., the cytoskeleton, were evident in the astrocytes of the marginal zone, particularly at the end foots of those glial processes in contact with the limiting membrane (Fig. 5).

Relationships between astrocytes and nerve fibers

In longitudinal sections of the optic nerve, it can be seen how longitudinal processes of the astrocytes appeared arranged following a parallel course closely associated with the nerve fibers. In the transverse sections, these longitudinal running processes can be seen as black points scattered between the nerve fibers (Fig. 3).

One thing that caught our attention was the presence of small incomplete rings that are part of these longitudinal cell processes and that are well to see in cross sections of the nerve. They seem to surround some fine cylindrical structure whose diameter correspond to that of the optic nerve axons, i.e., without myelin sheath, as is the case in the Ranvier nodes (Fig. 3). Although these

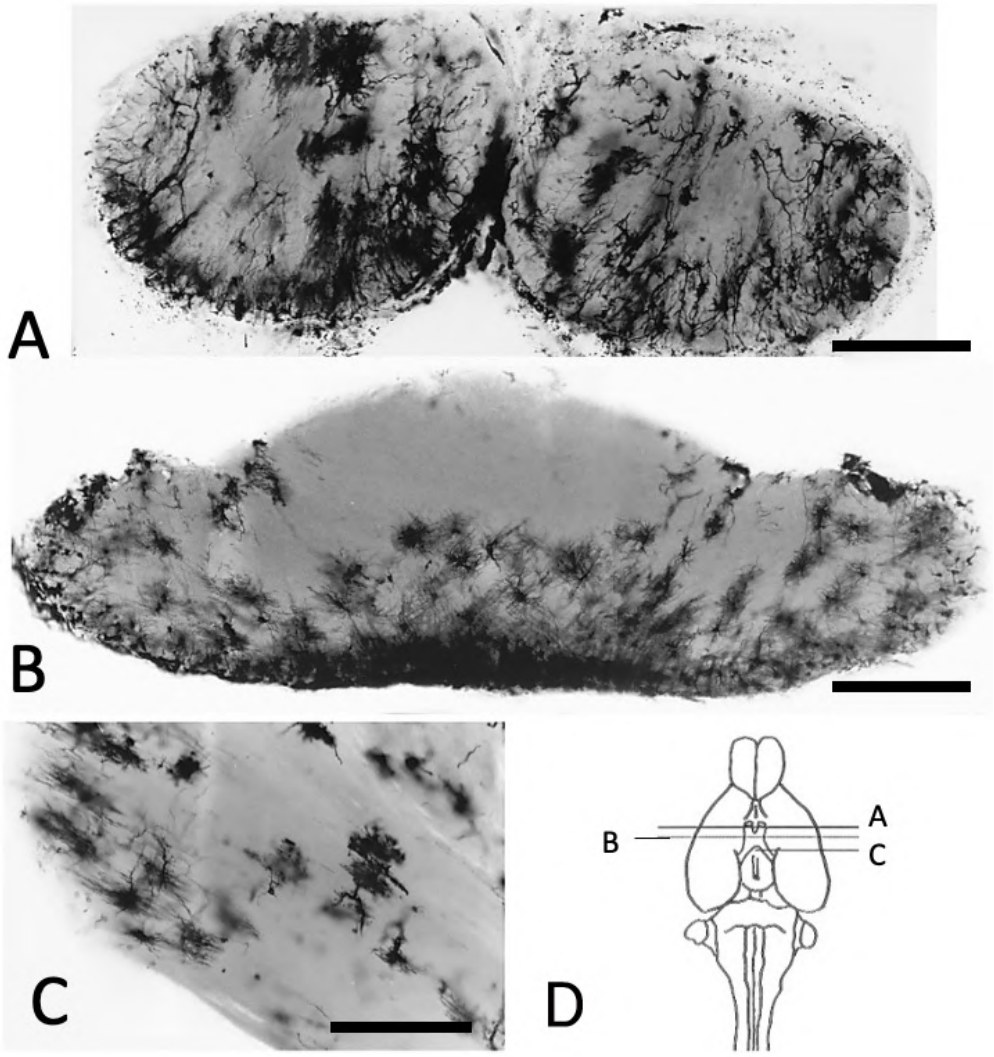

Fig. 1.- Cross sections of the optical paths at different levels (see diagram). **A)** both optical nerves shortly before forming the optic chiasm. Intensely stained astrocytes can be seen both in the margins and in the central part of the nerve (arrows). Bar= 100 µm. **B)** Cross section of the chiasm. Glial cells appear stained in the ventral part and lateral margins. Astrocytes are seen with great profusion of extensions (arrows). Bar= 100 µm. **C)** Cut through the optical tract. In the marginal zone astrocytes show great abundance of prolongations that insinuate along the nerve fiber fascicles (arrow). Bar= 100 µm. **D)** Diagram indicating the location of images A, B and C. At all path level's astrocytes stand out in black, with more impregnated cells in the periphery of the nerve, while in the tract the impregnation is more even.

rings are more frequently found associated to central astrocytes, the marginal astrocytes also have them in their prolongations.

These rings have been examined with Golgi-EM, and it has been confirmed that they are indeed extensions of astrocytes, and they coil around the axon at the Ranvier node level, establishing direct contact between astrocyte and axon membranes (Fig. 4). This description is valid for all the rings examined.

The astrocytes and their relationship with the limiting membrane

In the marginal zone, astrocytes project a good part of their radial running processes to the external limiting membrane, a basement membrane between pia mater and glial cells, where they end up forming terminal feet (Fig. 5).

In these processes, and just before the terminal foot, these processes have several small protuberances that inter-digitate with those of the neighboring astrocytic processes (Fig. 5A, insert). These protuberances appear interlaced in a close and compact way, which suggests a kind of cellular barrier attached to the external limiting membrane.

With the electron microscope, in these robust astrocytic extensions abundant gliofibrils were observed (Fig. 5).

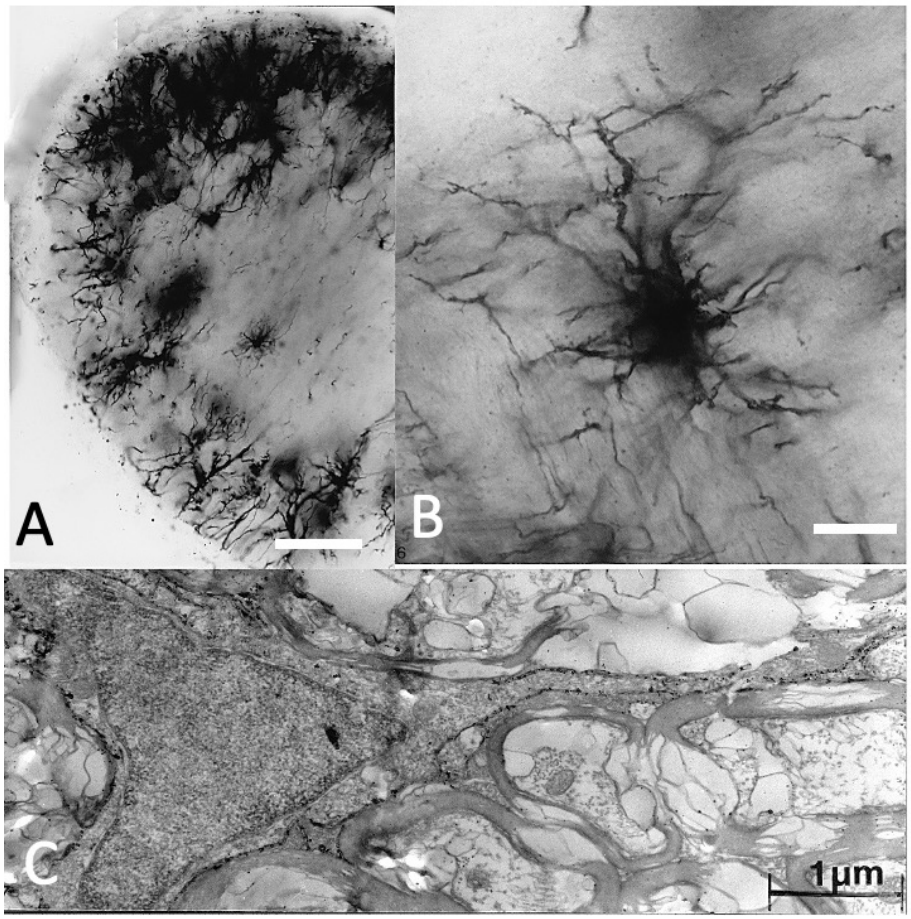

Fig. 2.- A) Cross section showing astrocytes in the marginal zone and in the center of the nerve. Bar= 100 µm. **B)** Astrocyte located in the center of the nerve with abundant ramifications of different orders. Bar= 10 µm. **C)** Golgi-EM gold-toning method. Note the fine deposit of metallic particles immediately below the plasma membrane. The cell nucleus (N) presents a homogeneous chromatin distribution, with soft condensations associated to the nuclear membrane.

Between the small protuberances of the glial processes, numerous gap junctions and direct apposition of cell membranes exist (Fig. 5).

Oligodendrocytes

These cells, following the Golgi procedure modified by the Rio-Hortega, appear very well stained and show a typical shape already described by this author (Rio-Hortega, 1928) with few processes, at least if compared to astrocytes, a fact that justifies its name.

In our samples, they appear sometimes isolated or one found many of them in relatively small areas. They were most frequently found in the intracranial portion of the optic nerve, in the chiasm itself, as well as some in the optical tract.

The soma of these cells is spherical or slightly oblong in shape, from which three or more main processes emerge, emitting several thinner branches that in the vicinity of the myelin sheaths of the nerve fibers can no longer be followed, as they are not impregnated, so that the relationship of oligodendrocytes with myelinated fibers of the optic nerve cannot be shown with Hortega's method (Fig. 6). Unlike astrocytes, in oligodendrocytes the soma impregnation is such that it allows to distinguish the cell nucleus as a clear or translucent field into the cell (Fig. 6).

The main processes are rather thin, and run on a plane more or less transversal to that of the nerve fibers, and as already mentioned, the secondary processes are unstained and practically undetectable close to the myelin sheath. In the most external part of the myelin sheath to which the cellular process is associated, there is still a certain amount of cytoplasm, so that the cell membrane there could be detected in thin sections with a TEM. However, such structural

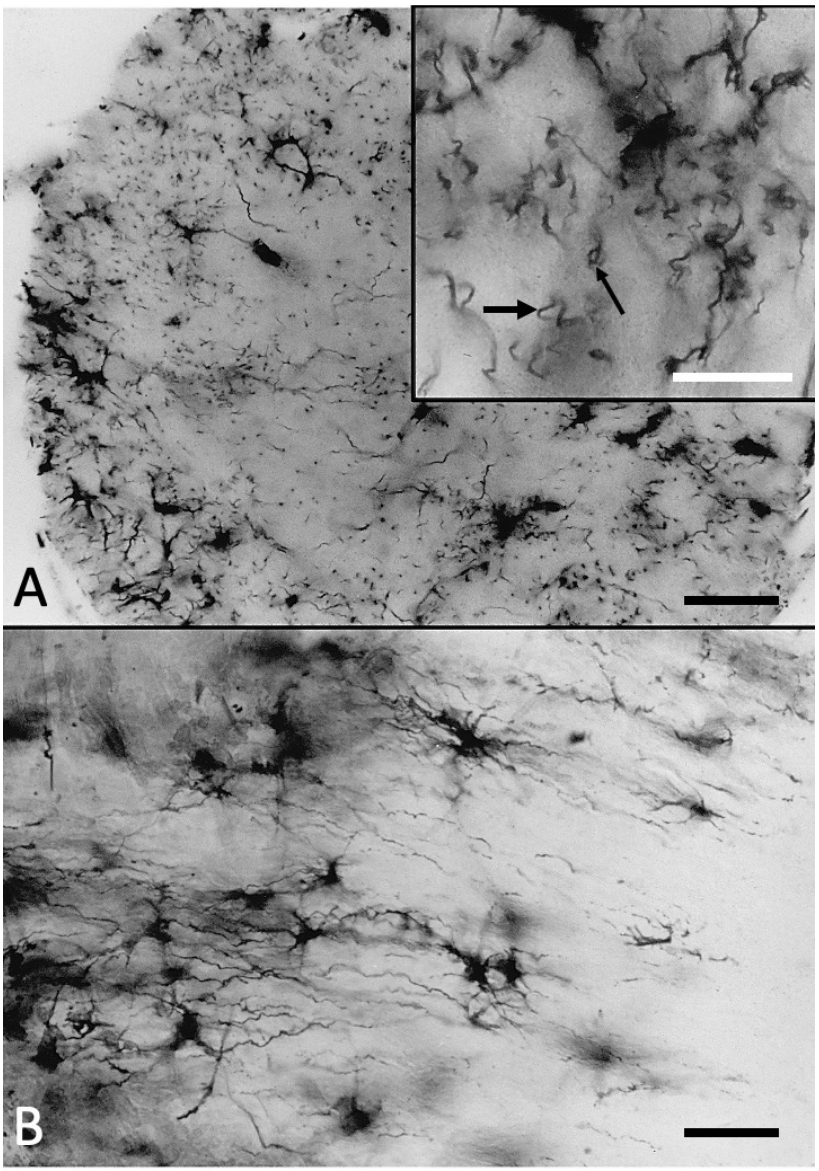

Fig. 3.- Cross section **(A)** and longitudinal section **(B)** of the optic nerve. In A there are abundant black points corresponding to transversely cut astrocyte processes. Bar = 50 µm. In the insert of A, small complete and incomplete ring formations can be distinguished from the astrocyte extensions (arrows). Bar= 20 µm. In B we can see processes of astrocytes oriented longitudinally and with a parallel course to the optic nerve fibers. Bar= 50 µm.

details could not be visualized in impregnated material.

In our specimens we have not observed specific relationships of the oligodendrocytes with the vessels, also not with the external limiting lamina of the optic nerve.

DISCUSSION

In the first decades of the past century, probably the first author who has shown images of the astrocytes of the optic nerve in its intracranial portion has been Ramon y Cajal, in the context of his retina investigations (1909). More recently, images of astrocytes of the optic nerve have been published, but applying variants of the Golgi method different from ours (Miller et al., 1989; Reichenbach et al, 1992). With the variant of the Golgi method described by Rio-Hortega (1928), high quality impregnations of the macroglia cells of the albino rat optic nerve have been achieved, showing the preparations a very clean background and without contaminating precipitates (Mestres, 1988).

The astrocytes described in the present study exhibit a morphology known of classical fibrous astrocytes, cells existent in all locations of the white matter of the central nervous system (Montgomery, 1994; Lundgaard et al. 2014; Li et al., 2016). The distinction made here between central and marginal astrocytes is simply a

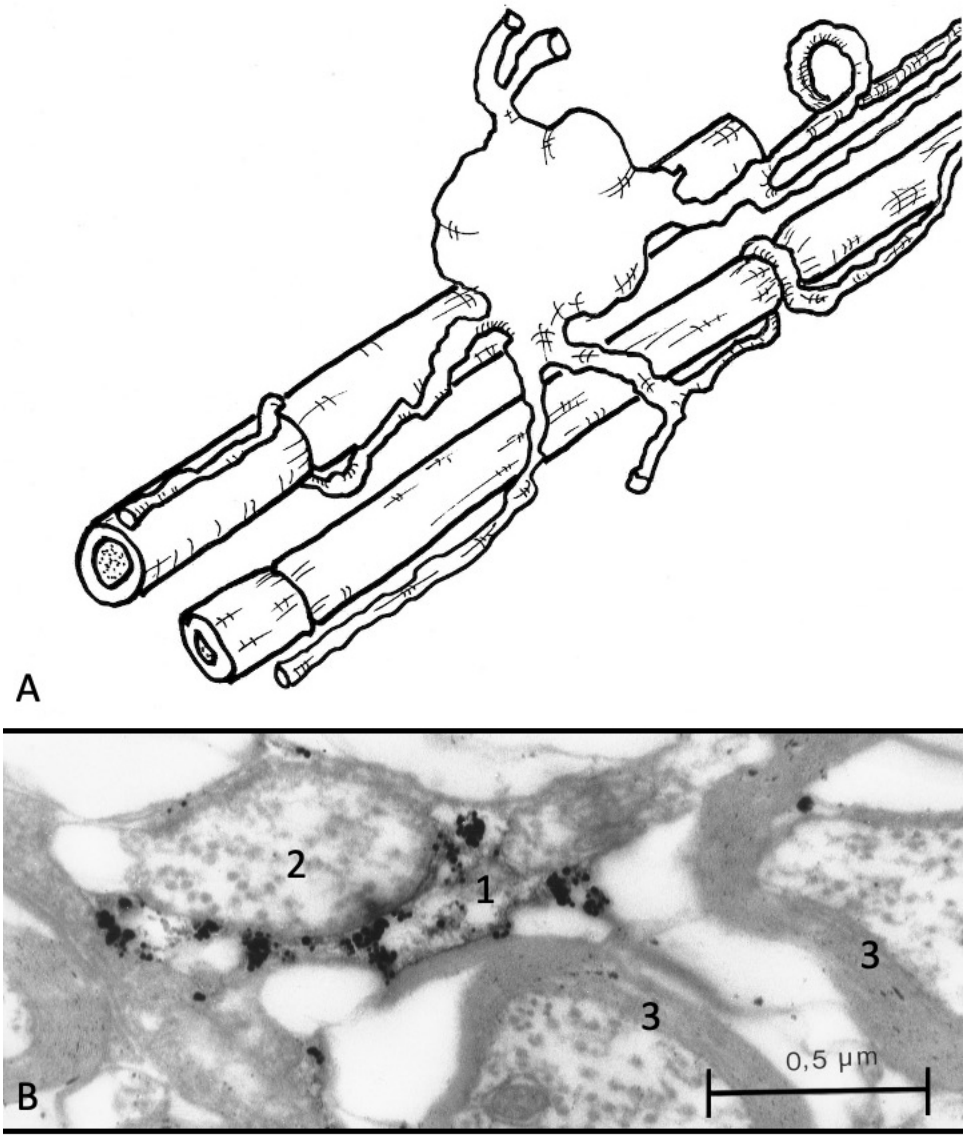

Fig. 4.- A) Diagram showing the relationship between the ring formations and the Ranvier nodes of the optic nerve fibers. **B)** Golgi-EM with gold toning of an astrocyte process (1) in contact with the axon in the Ranvier node (2). Note the lack of myelin sheath at that level and compare with (3).

subterfuge for the presentation of our results in a understandable manner, but it should be noted that the morphological differences between one and the other are really slight if at all, and may merely reflect the spatial adaptation of the cells to anatomical features of the area.

A technique that has made possible to obtain completely labeled astrocytes in the optic nerve is the intracellular micro-injection of markers such as HRP or fluorescents such as Lucifer Yellow (Butt et al., 1994). For example, HRP labeling produces images with high contrast, and in them the cell processes can be distinguished accurately without overlapping with those of the neighboring cells, and the overhang of the processes can be appreciated in their entirety (Butt and Ransom, 1989). Such a situation also occurs in the Golgi preparations, although less frequently and in principle without being able to directly influence the result, unless the protocol of the method is fortunately modified.

In our preparations we have found astrocytes, whose processes establish contact with blood vessels, are approaching to the fibers of the optic nerve and with their terminal feet contact the subpial basement membrane, which separates the parenchyma of the nerve from the pial sheath that surrounds it. This description agrees with those based on HRP microinjections and also with fluorescent tracers, techniques with which the

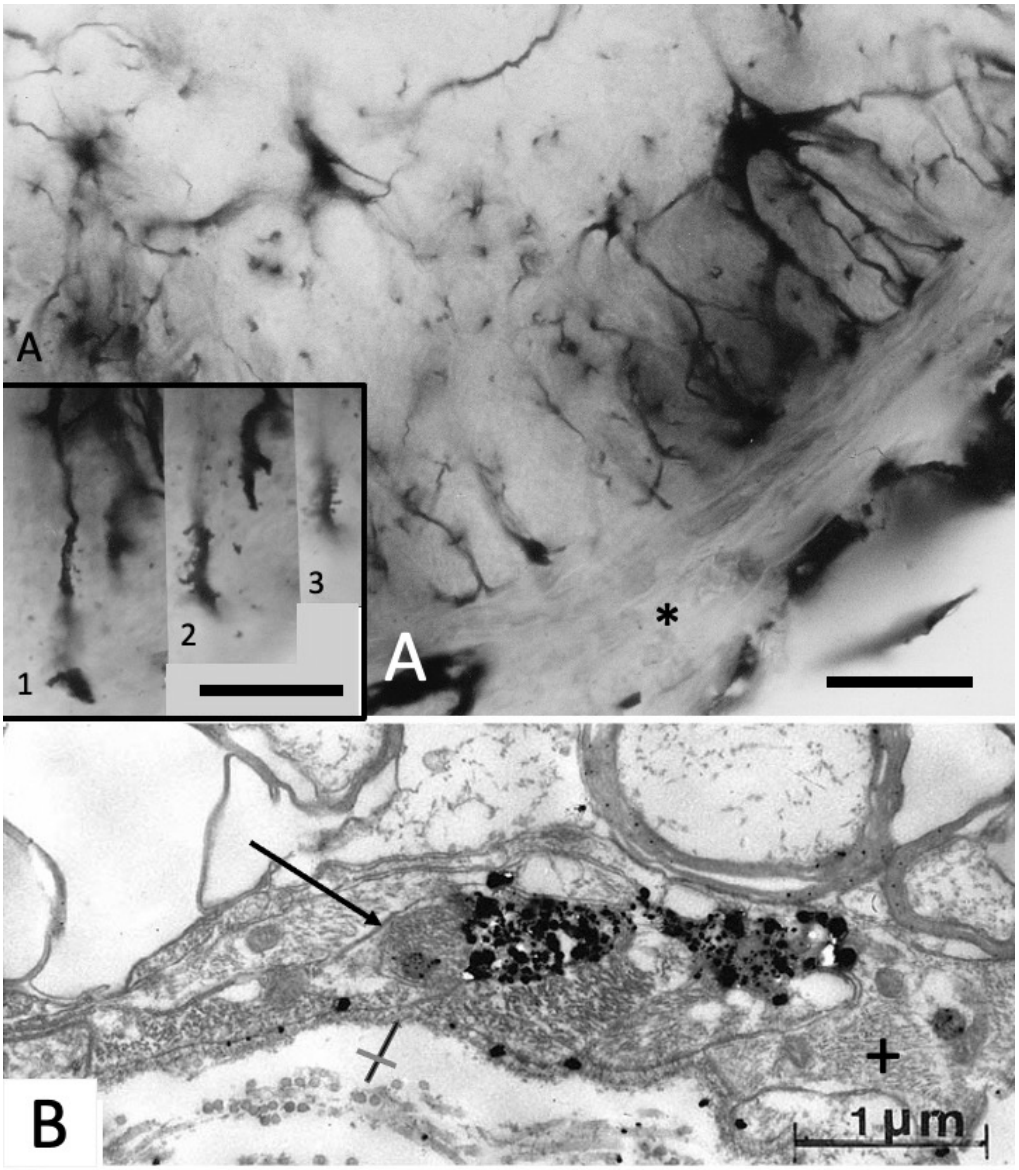

Fig. 5.- Marginal zone of the optic nerve. **A)** Astrocytes with feet in contact with the limiting membrane and the meningeal envelope (*). Bar= 40 µm. Insert: Three focusing planes (1, 2, 3) of an astrocyte end foot showing fine spines or protrusions that articulate with neighboring similar formations. Bar= 20 µm. **B)** Feet of astrocytes in contact with the limiting membrane (cross arrow) with gliofilaments (+) and gap junctions between them (arrow). Collagen from the meningeal layers is visible at the bottom.

known uncertainty in terms of results of the Golgi method can be faced with great advantage (Butt and Ransom, 1989, 1993).

Studies by Butt's group state that astrocytes from any location within the nerve establish the contacts mentioned above, but do not confirm the differences in the contact patterns proposed by other authors, who postulate the existence of two different types of astrocytes in the optic nerve, which would be distinguished by their relationship with other structures such as blood vessels and pial surface, a hypothesis that in view of the results of other authors and of our own does not seem sustainable at all (Miller et al., 1989; Butt et al., 1994).

On the other hand, the optic nerve is nothing more than a special compartment of the white matter of the brain, in which there are only astrocytes of the fibrous type and oligodendrocytes, later forming the myelin sheaths around the corresponding axons (Peters et al., 1976). However, studies with specific antibodies such as A2B5, which binds to surface markers such as certain gangliosides, have revealed the existence of astrocytes with positive marking and others that are negative (Raff, 1989). Based mostly on immunocytochemical studies with A2B5 and investigations on the cell linage, the existence of two different astrocytes, type- 1, related with the classical protoplasmic astrocyte, and type-2 related with the

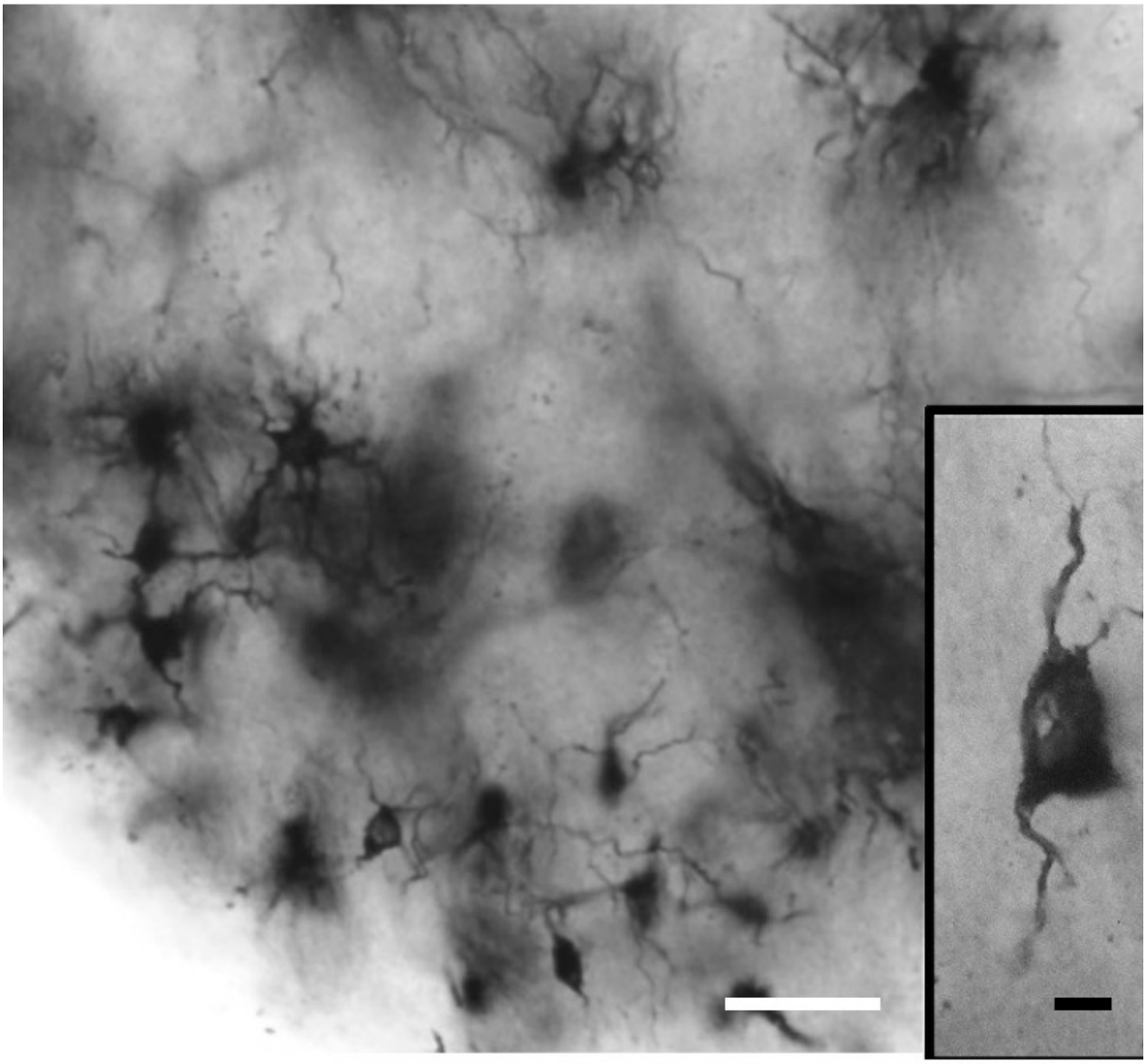

Fig. 6.- Field of the optic nerve showing astrocytes (upper part) and oligodendrocytes (lower part). Bar= 50 µm. Insert: Detail of an oligodendrocyte. Bar= 20 µm.

classical fibrous astrocyte, in the optic nerve has been postulated, which, without a doubt, would be a very unique fact, considering the general cell composition of the white matter (Scolding et al., 1999). On the one hand, the A2B5 antibody binds to gangliosides that are expressed in glial precursor cells and also in various cancers, and in some cases the expression is transient, particularly under in vitro conditions and at certain moments of development (Haas et al., 2012). At this point, the question of the reliability of this labeling seems at least justified. As has already been said, a histological classification of the astrocytes of the optic nerve in terms of anatomical relationship, at has been postulated by others, does not seem to hold up in light of Golgi studies, and especially of investigations with the tracer microinjections technique (Butt and Ransom, 1989, 1993).

Certainly, the astrocytes located near the surface of the nerve present well visible radial extensions that end in the subpial basement membrane; but also the astrocytes located more deeply within the nerve present such contacts, which agrees with what has been observed in our preparations and by other authors (Butt and Ransom, 1989; Butt et al., 1994).

In the present study, it has been seen how astrocytes form a glial foot zone next to the subpial basement membrane, where such feet are closely intertwining and forming something

reminiscent of the outer glial zone of the cerebral cortex (Braak, 1975). Similar to the cortex, in the optic nerve the outer glial feet contains abundant intermediate filaments, 10 nm thick filaments surely GFAP positive (Sun et al., 2009), the glial end feet appearing interconnected by numerous gap junctions (Braak, 1975). In the optic nerve, there exist gap junctions between astrocytes, and also between astrocytes and oligodendrocytes, but not between oligodendrocytes (Orthmann-Murphy et al. 2008). Astrocytes located rather to the center of the nerve generally have thin and long processes, which end by means of glial feet on several anatomical structures, such vessels and pial surface.

Our description of the astrocytic processes and their relationship with Ranvier's nodes is in direct agreement with the previous investigations dealing with the optic nerve and the spinal cord (Hildebrand, 1971; Raine, 1984; Sims et al., 1985). The gold-toning technique (Fairén et al., 1974; Mestres and Schneider, 1997), applied for the first time to visualize in TEM glial cells of the optic nerve stained according with the del Rio-Hortega method, allows the observation of intracellular details of the astrocytes, as well as the relationship of their processes with the Ranvier node. These observations are in agreement with those obtained in transmission electron microscopy with or without combination with tracer techniques (Butt et al., 1994). What could be designated as a new observation fact are the rings that form the astrocyte processes around the Ranvier node, which in this way had not been visualized until now (see interpretation diagram in Fig. 5).

The oligodendrocytes have been also well impregnated applying the Golgi-Hortega method. In these cells, only the main processes have been stained, getting lost or finishing in the proximity to the myelin sheaths. However, studies with micro-injection techniques show in injected oligodendrocytes numerous extensions of longitudinal course and therefore parallel to the nerve fibers (Hildebrand, 1971; Butt and Ransom, 1989). It is likely that these tracers label compartments due to an intracellular diffusion of the same in the inner and outer cytoplasmic border of the myeline sheath after wrapping around the axon, hence their appearance as if they were processes of oligodendrocytes running alongside the myelinic sheaths, as if they were cellular extensions, which they are not in fact. The Golgi method fails to impregnate these cytoplasmic zones of the myelin sheath, and only the rather short portions of the oligodendrocyte processes connecting cell body and myelin sheath appear stained.

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