

# Ultrastructural Findings in Human Spinal Pia Mater in Relation to Subarachnoid Anesthesia

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We examined ultrastructural details such as the cellular component and membrane thickness of human spinal pia mater with the aim of determining whether fenestrations are present. We hypothesized that pia mater is not a continuous membrane but, instead, that there are fenestrations across the pial cellular membrane. The lumbar dural sac from 7 fresh human cadavers was removed, and samples from lumbar spinal pia mater were studied by special staining techniques, immunohistochemistry, and transmission and scanning electron microscopy. A pial layer made by flat overlapping

cells and subpial tissue was identified. We found fenestrations in samples from human spinal pia mater at the thoracic-lumbar junction, conus medullaris, and nerve root levels, but these fenestrations did not appear at the thoracic level. We speculate whether the presence of fenestrations in human spinal pia mater at the level of the lumbar spinal cord and at the nerve root levels has any influence on the transfer of local anesthetics across this membrane.

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**T**he central nervous system has three covering membranes known as meninges: dura mater, arachnoid mater, and pia mater. Pia mater is the innermost of these membranes, closely investing the brain, spinal cord, and nerve roots within the spinal canal. However, it has not attracted the interest of anesthesiologists to the same degree as has dura mater. Pia mater is the only interposing membrane between the subarachnoid injection of local anesthetics and lumbar spinal nerve axons; therefore, it may act as a physiological barrier, influencing the onset time or latency of subarachnoid blocks (1). Factors known to have an effect on the onset of such blocks are, for instance, nerve root diameter, local anesthetic pKa, and tissue pH.

We hypothesized that the pia mater, instead of being a continuous membrane, would have fenestrations. Pia mater was previously examined in humans

at the cranial and spinal levels (2–5) and was described as a continuous layer of flat pial cells. Studies of pia mater from animals, however, found fenestrations within its thickness (6). We studied the ultrastructure of pia mater in human lumbar and thoracic spinal meningeal samples and reviewed morphological aspects, such as the thickness of its cellular layer and subpial tissue, as well as whether fenestrations were present throughout this membrane.

## Methods

Approval from the research ethics committee and family consent for the donation of organs, for autopsy, and for procedures included in this research were obtained. The dural sac and its contents at the thoracic and lumbar level were removed from 7 fresh human cadavers between 48 and 78-yr-of-age. Cause of death excluded chronic medical or surgical illness.

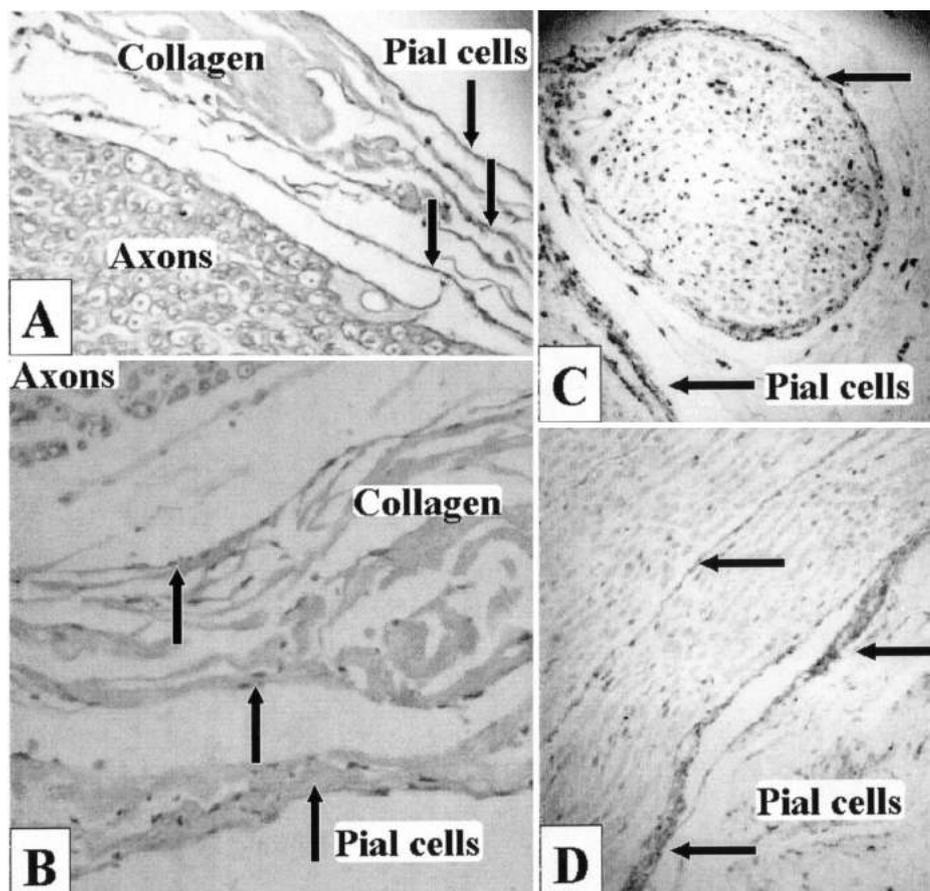
After organ donation in three cadavers and autopsy in 4 of them, anterior laminectomies were performed, and the dural sac was removed without damaging its contents at the thoracic and lumbar sites. The time from death to the fixing of samples was 1 to 8 h.

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**Figure 1.** Pial cells from nerve root samples (light microscopy). A and B, A nerve root wrapped by five concentric cellular layers. Collagen fibers were seen with interposing between them. Transversal cuts were stained with Masson's trichrome. Axons = myelinated axons; collagen = collagen fibers. C and D, Pial cells were found around the nerve root and identified by immunohistological techniques. Transverse and longitudinal cuts were stained with immunostaining (streptavidin-biotin-peroxidase technique). The arrows show nuclei from pial cells.

We dissected the lower thoracic and lumbar spinal cord and as far as the conus medullaris, making a vertical incision along the spinal cord midline to obtain anterior and posterior spinal cord and nerve root segments. Thereafter, we cut samples measuring 20 mm in length for examination using scanning electron microscopy (SEM), alternating with samples of 4 to 5 mm to be studied by histological techniques.

### Light Microscopy

Special histological staining methods were used to identify structures under light microscopy. Electron microscopy techniques were used to observe ultrastructural details from samples.

Samples to be observed under the light microscope were fixed with 10% buffered formaldehyde; after fixation, cuts of 4 mm in thickness were made with a microtome. The samples were then stained with different dyes under standard conditions for hematoxylin-eosin techniques, Masson's trichrome, orcein, and periodic acid-Schiff Alcian blue to a pH of 2.5.

Immunostaining was performed on paraffin-embedded tissue after the streptavidin-biotin-peroxidase technique. Sections with a thickness of 4 to 6  $\mu\text{m}$  were cut, air-dried for 15 min, and heat-fixed at 58°C–60°C overnight and then at 100°C for 10 min. Slides were

deparaffinized with xylene, dehydrated with progressive use of alcohol, and then washed in distilled water. Antigen retrieval was achieved with a pressure cooker for 90 min. Treating the slides with  $\text{H}_2\text{O}_2$  for 10 min eliminated endogenous peroxidase activity. Afterward, the slides were incubated for 30 min with diluted primary antibodies obtained from mouse: prediluted epithelial membrane antigen (clona, ZCE, 113) and prediluted anti-human macrophage (CD68; clona KP1) (Master Diagnostica, Granada, Spain). The technique was performed in an automated immunohistochemical Steiner (LAB Vision Corp., Master Diagnostica). Aminoethylcarbazole substrate solution was used to make the peroxidase reaction visible. Sections were counterstained with hematoxylin (Sigma), dehydrated in alcohol, cleared in xylene, and mounted.

### Transmission Electron Microscope (TEM)

The specimens were fixed for 4 h in a solution of glutaraldehyde 2.5% and a buffered phosphate solution to a pH of 7.2–7.3. They were later fixed with a solution of 1% osmium tetroxide for 1 h. The specimens were dehydrated with increasing concentrations of acetone in water and soaked in resin epoxy (Epon 812). The resin was polymerized to 70°C for 72 h. To

identify the sites where ultrathin cuts would be made, control group slides were dyed with Richardson's methylene blue and observed under light microscopy. Ultrathin slides, 70 nm thick, were cut with an ultramicrotome and treated with 2% acetate of uranilo solution, as well as Reynold's lead citrate solution. The specimens were then observed under a Zeitz EM 902 transmission electron microscope (TEM).

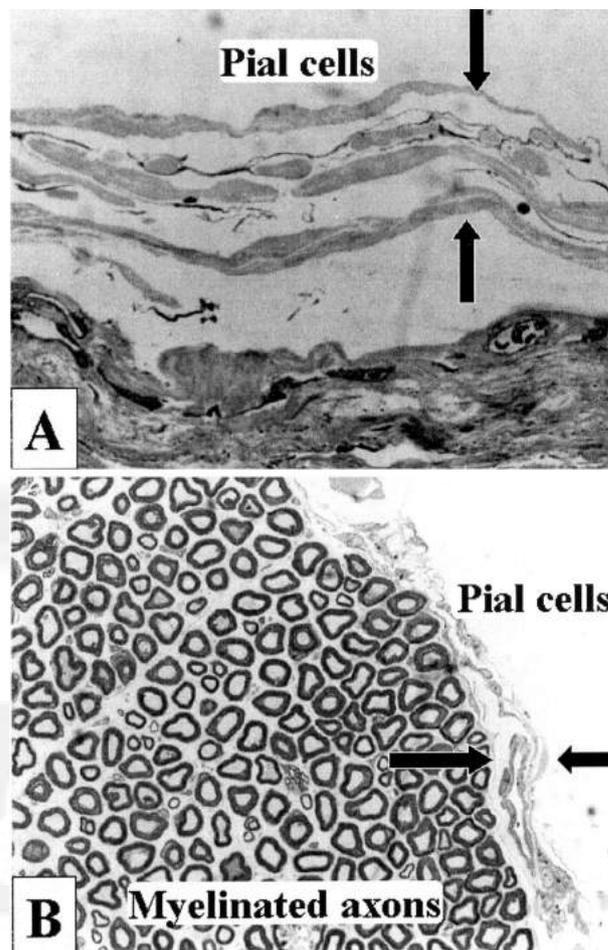
### SEM

The samples were fixed by immersion in a solution of 2.5% glutaraldehyde and buffered phosphate solution (pH of 7.2-7.3). They were later dehydrated in increasing concentrations of acetone and pressurized under carbon dioxide (Balzers CPD 030 Critical Point Dryer; Bal Tec AG, Fürstetum, Liechtenstein) to reach the critical point. Tissue samples measuring 20 mm in length were placed over slides with a diameter of 25 mm. A carbon layer was then deposited on the samples to a thickness of less than 20 nm and covered with a gold microfilm (Vaporization Chamber SCD 004; Balzers Sputter Coater). Afterward, the samples were observed under a JEOL JSM 6400 SEM (JEOL Corp. Ltd., Tokyo, Japan). Approximately 70% to 80% of the tissue belonging to spinal cord and nerve roots was examined under SEM. Approximately 20% to 30% of the tissue was examined by histological techniques. Only a small amount of tissue was rejected because of poor quality.

### Results

To observe ultrastructural details of human pia mater, we used TEM. SEM enabled us to observe details of the pia mater's surface, including the presence of fenestrations at the level of the lumbar spinal cord, conus medullaris, and nerve roots; such fenestrations were not found in pia mater at the thoracic level. Each component related to pia mater was identified by using special staining techniques (SST) or immunohistochemical techniques (IHT). Using Masson's trichrome staining, we found large amounts of collagen fibers. Orcein staining identified a few elastic fibers. Fibroblasts were found with hematoxylin-eosin; an amorphous intracellular substance, periodic acid-Schiff Alcian blue; pial cells, arachnoid cells, and macrophages (IHT). The results obtained by SST, IHT, TEM, or SEM referring to the same structure will be described by indicating the method in parentheses.

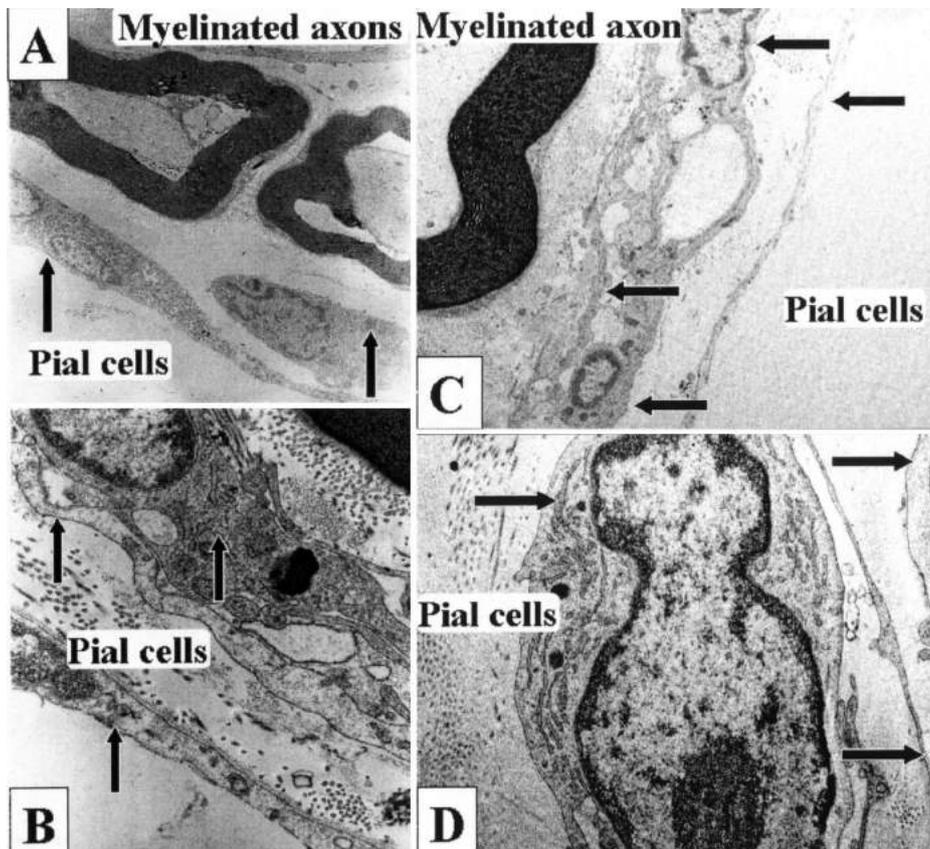
Pia mater is arranged in a cellular layer shaped by flat pial cells investing the spinal cord, anterior median sulci, conus medullaris, filum terminale, and nerve roots (SST, IHT, and TEM) (Figs. 1-3). Subpial tissue containing mainly collagen fibers separates the pial cellular layer from neuroglial cells (SST) (Fig. 1). Some samples observed under SEM



**Figure 2.** Pial cells from the nerve root were stained with Richardson's methylene blue (light microscopy); four pial concentric layers are identified within the arrows. The nerve root has mostly myelinated axons. A, Longitudinal cut; B, transverse cut.

showed a detachment between the pial cellular layer and subpial tissue, favoring the observation of these structures (Fig. 4); the space originated by this detachment is considered an artifact because it was not observed with any other technique (SST, IHT, or TEM) (Figs. 1-3).

The pial cellular layer contains flat overlapping pial cells (TEM) (Fig. 3) with an oval-shaped nucleus and a small nucleolus (TEM) (Fig. 3). Cytoplasmic structures, such as mitochondria, rough-surface endoplasmic reticulum, and vacuoles, were identified (TEM) (Fig. 5). Under TEM, pia mater was seen as a delicate and, apparently, continuous cellular layer joined by desmosomes and other specialized junctions (Fig. 3). At the level of the nucleus, pial cells had a thickness of 4  $\mu\text{m}$ ; at other levels, pial cells measured on average 0.5-1  $\mu\text{m}$  (TEM) (Fig. 3). Under SEM, a tridimensional view of the pia mater's surface was obtained; it appeared smooth and bright (Fig. 6). With SEM, we noticed the presence of blebs (cytoplasmic protrusions) on the cytoplasmic



**Figure 3.** Pial cells at the level of the nerve root, marked by arrows. A, Two pial cells in detail. A cytoplasmic elongation from a pial cell is superimposed over its neighboring cells. Two myelinic axons are close to pial cells; between them, collagen fibers are found as part of the subpial tissue (magnification 3000 $\times$ ). B, Pial cells' plasmatic membranes and their junctions. The arrows in the picture show three pial cells; the pial cell placed above is thicker because the cut was made through its nucleus and appears closest to subpial tissue (magnification 12,000 $\times$ ). C, Superimposed pial cells and their cytoplasm. Subpial tissue and myelinic axons are also seen (magnification 3000 $\times$ ). D, Pial cell nucleus and collagen fibers from subpial tissue placed to the left (magnification 12,000 $\times$ ; transmission electron microscopy).

membrane of pial cells, although no microvilli (cytoplasmic elongations) were found. Some samples showed single cytoplasmic outgrowths placed at the junction of two pial cells (SEM).

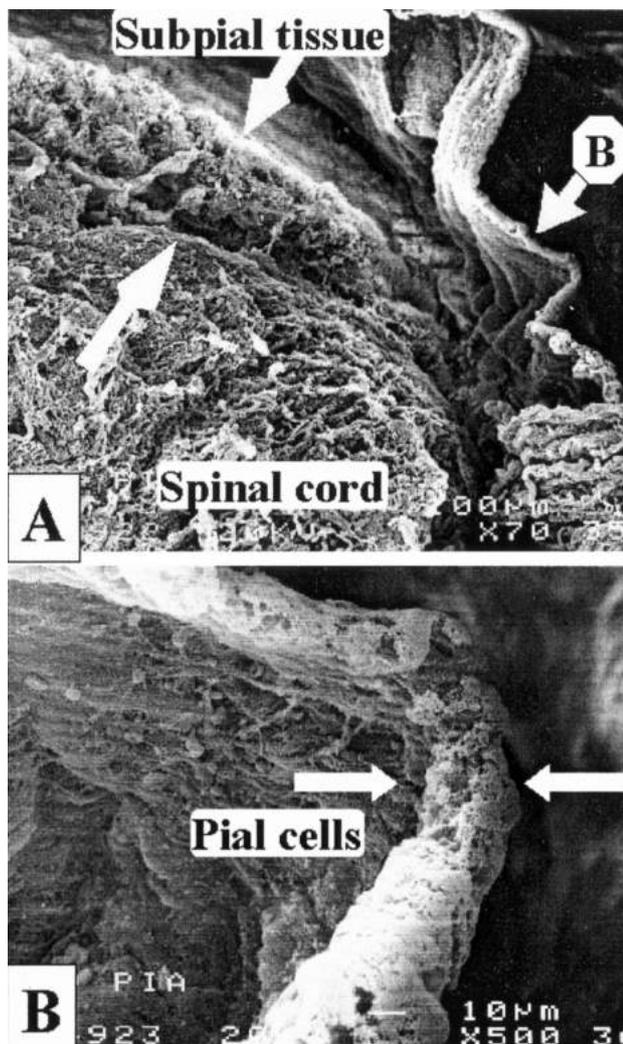
The pial cellular layer at the level of the spinal cord is three to six pial cells thick (8–15  $\mu\text{m}$ ) (TEM and SEM) (Figs. 3 and 4); these measurements were similar at the thoracic, lumbar, and conus medullaris levels. On the nerve roots, the pial cellular layer has a thickness of two to five pial cells (3–8  $\mu\text{m}$ ). In some spinal cord and nerve root samples, two or four concentric cellular layers were seen with interposing collagen fibers between them (SST and TEM) (Figs. 1 and 3).

At the level of the spinal cord and nerve roots, the subpial tissue contains the following structures: large amounts of collagen fibers (SST, TEM, and SEM) (Fig. 2–4), a few elastic fibers (SST and SEM), amorphous intracellular substances (SST), small vessels (TEM), fibroblasts (SST and TEM), and macrophages (IHT and TEM). Collagen fibers appear oriented in different directions, and each fiber measures on average 0.1  $\mu\text{m}$  in diameter (SEM and TEM). The structure, composition, orientation, and thickness of these collagen fibers look similar in all the samples. Elastic fibers are 1.4 to 2  $\mu\text{m}$  in diameter and have a rough surface (SEM). Subpial tissue has a thickness of 130–200  $\mu\text{m}$  at the thoracic and lumbar levels and decreases at other

levels to 80–90  $\mu\text{m}$  (conus medullaris), 40–50  $\mu\text{m}$  (initial portion of cauda equine), and 10–12  $\mu\text{m}$  (nerve roots) (SEM) (Fig. 4).

We examined samples under SEM and found fenestrations on the pia mater's surface in the thoracic-lumbar junction and conus medullaris (SEM). The most pial fenestrations were found at the level of the conus medullaris in 5 of 7 cadavers. Most of these fenestrations appeared sited in its distal portion. However, at the level of T8 and T9, these fenestrations were not found (SEM).

In one cadaver, only a few fenestrations were seen at the level of the spinal cord, whereas in another, these fenestrations were not found at any level of the spinal cord (SEM). The fenestrations have circular, ovoid, or elliptical shapes (SEM), and their measures vary; most of them have a length of 12–15  $\mu\text{m}$  and a width of 4–8  $\mu\text{m}$  (SEM) (Figs. 6 and 7). At the level of the nerve roots, pia mater has similar and variable numbers of fenestrations, although these are smaller: 1–4  $\mu\text{m}$  in diameter (SEM) (Fig. 7). Some nerve root samples did not show fenestrations. On examination, the fenestrations seemed symmetrically arranged, with curved and smooth edges. There was no evidence of radial shearing, fragmentation, or tissue folding in them (SEM). To evaluate their true nature, we applied forces in different directions over thoracic

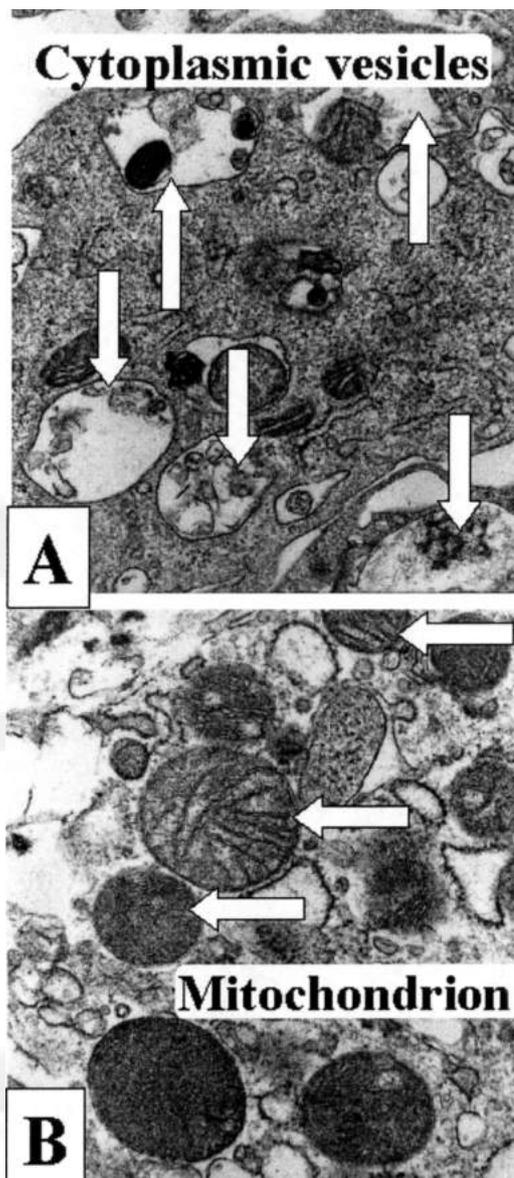


**Figure 4.** Pia mater at the level of the spinal cord. A, Fraction of the pial cellular layer, presumably detached during dissection. Subpial tissue with collagen fibers is placed between the pial layer and spinal cord. The space seen between the pial layer and the subpial tissue is an artifact. The "B" arrow marks the area that has been magnified in (B) (scanning electron microscopy; magnification 70 $\times$ ). B, Pial cellular layer. The arrows delimit the pia mater thickness (magnification 500 $\times$ ; scanning electron microscopy).

samples that did not contain fenestrations. The resulting tears and lacerations did not look similar in any way to the fenestrations found in other samples (conus medullaris).

We found a core of collagen fibers and amorphous intercellular substance in the trabecular arachnoids, covered by arachnoid cells joined together (TEM). Where the trabecular arachnoids meet the pial surface, arachnoid cells that come into contact with pial cells can no longer be differentiated (IHT). At the level of spinal cord and nerve roots, collagen fibers from subpial tissue extend without interruption into the trabecular arachnoids (TEM).

Only a few macrophages were seen in our samples; they were found within the subpial tissue amid pial cells

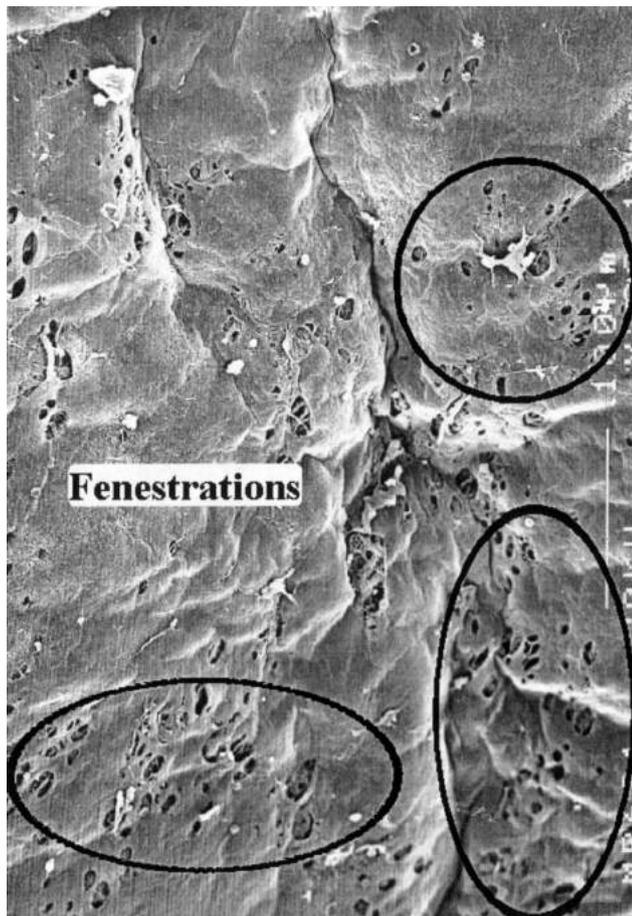


**Figure 5.** Pial cells from nerve root samples; the figure shows their cytoplasm. A, Arrows showing numerous vesicles and their content (magnification  $\times 20,000$ ). B, Arrows pointing toward a few mitochondria (magnification 30,000 $\times$ ; transmission electron microscopy).

and their basal membrane (IHT and TEM). In only two samples did we find macrophages on the pial surface in contact with the subarachnoid space (IHT and TEM). Macrophages lack long cytoplasmic processes; instead, they have membrane-bound inclusions and vacuoles (TEM). These vacuoles are found in outer parts of the cytoplasm and vary in size and shape (TEM). Pial cells looked similar to fibroblasts but showed few mitochondria and little endoplasmic reticulum (TEM).

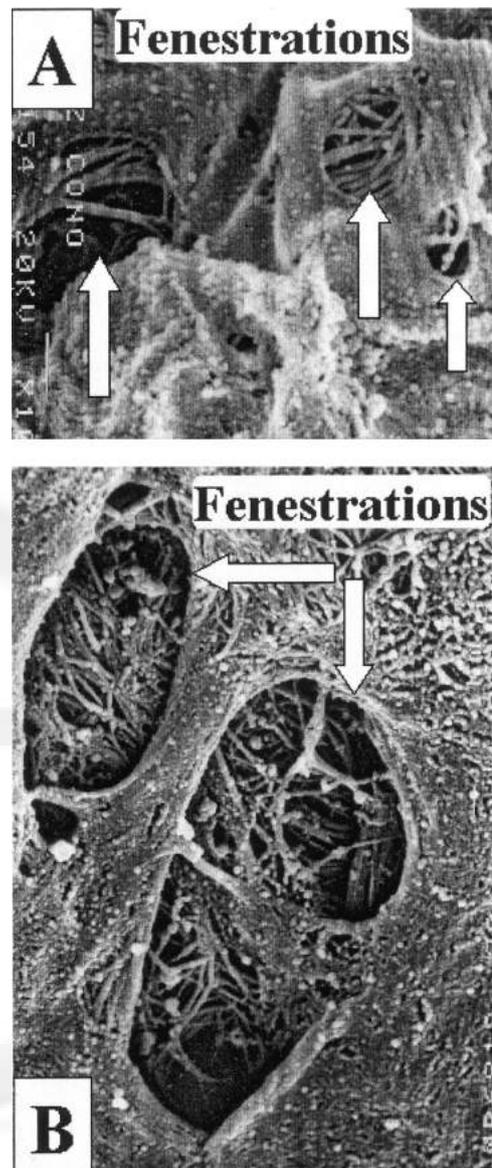
## Discussion

Our aim was to study the ultrastructural anatomy of human spinal pia mater to elucidate the nature of pial



**Figure 6.** Fenestrations found in the pial cellular layer at the level of the spinal cord. Numerous fenestrations appear in the picture. Sites with more fenestrations are circled (magnification 220 $\times$ ; scanning electron microscopy).

constituents that could affect the transit of local anesthetics across this membrane. We observed the presence of fenestrations within the pial cellular layer from the human lumbar spinal cord at the level of the conus medullaris and the nerve roots (SEM). Morse and Low (7), Cloyd and Low (6), and Krahn (8) described fenestrations in the pia mater of animals at the level of the conus medullaris in the spinal cord (6,7) and in pia mater around the entrance of spinal blood vessels (8). To familiarize ourselves with the structure of fenestrations, we examined them in the spinal pia mater from cats (9). Compared with those in cats, human fenestrations look alike. Most were found at the level of the conus medullaris in the lumbar spinal cord, and only a few were found in the thoracic-lumbar junction. No fenestrations were seen at the higher thoracic levels, as was also noted by Nicholas and Weller (2). Samples from one cadaver did not have fenestrations. We also observed many fenestrations, smaller and randomly distributed, within pia mater that wrapped around spinal nerve roots in their way across the subarachnoid space; however, not all samples from nerve roots



**Figure 7.** Fenestrations in more detail. Looking through the spaces circled by fenestrations, collagen fibers from the subpial tissue can be seen. A, Pia mater at the level of the nerve root (magnification 10,000 $\times$ ). B, Pia mater at the level of the spinal cord (magnification 4500 $\times$ ). Fenestrations in (A) and (B) have smooth edges, without any signs of tearing or damage (scanning electron microscopy).

presented fenestrations. Some samples of equivalent nerve roots from different cadavers did not show fenestrations, whereas other samples did.

Because of the absence of pial cells inside fenestrations, the basal membrane is the only continuous structure interposing between neural cells and cerebrospinal fluid. The presence of pial fenestrations is probably relevant to pia mater's lack of a physiological barrier effect.

We speculate that a fenestrated pia constitutes a factor to be considered in relation to the variability in onset time or latency of subarachnoid blocks. The fact

that most of the fenestrations were found at the level of the conus medullaris might have some influence in the almost-instant onset of action of some subarachnoid blocks. Further research is needed to clarify the real significance of our finding. Measuring subpial tissue thickness at different levels, we found that at the level of the nerve roots, the subpial tissue has the smallest diameter; its thickness increases progressively along the conus medullaris toward the lumbar spinal cord. In contrast to pia mater, the arachnoid membrane is thicker and contains larger numbers of cells joined by numerous specialized unions (10).

The noncontinuous layers defined by other authors do not resemble the fenestrations observed in our study (11,12). Haller and Low (12) and Kaar and Fraher (11) described a trabecular arachnoidal component as a "web-like layer," or noncontinuous layer, within the subarachnoid space that extended from the arachnoid inner surface to the surface of the pia mater at the spinal cord and nerve root levels. This "web-like layer" was located on the surface of the pial cellular layer. However, looking through the spaces encircled by fenestrations, we observed collagen fibers beneath the pial cellular layer that belonged to the subpial tissue (SEM) (Fig. 7).

Bernards (13) suggested that the pial and arachnoid cells that give shape to subarachnoid trabeculae are histologically identical, namely, "leptomeningeal cells." In our study, we also saw overlapping between pial cells and arachnoid cells enclosing arachnoid trabeculae (TEM and IHT). We describe in humans, as previously done in rats (11), how subpial tissue and arachnoid trabeculae share collagen fibers that extend without interruption from one structure to the other. Kaar and Fraher (11) also mentioned the continuity of collagen fibers between the pia and endoneurium in spinal nerve roots from rats. In our study, some spinal nerve root samples showed 2 or 3 pial cellular laminae and collagen fibers between laminae. This arrangement is similar to the perineural disposition of peripheral nerves (14,15). It is possible that the perineural layers that cover each nerve root fascicle originate from leptomeningeal cells (pial and arachnoid cells). Fraher and McDougall (16) identified macrophages adhering to the pial surface; Krahn (8) and Malloy and Low (17) found them dispersed within the pia mater and nerve roots, and we saw them in the subpial tissue.

In summary, we studied the ultrastructural anatomy of the pia mater, such as pial cells, membrane thickness, and subpial tissue, at different levels of the thoracic and lumbar spinal cord and nerve roots. We found fenestrations in samples from human spinal pia mater at the thoracic-lumbar junction, conus medullaris, and nerve root levels. These fenestrations were

mainly distributed along the conus medullaris and spinal nerve roots. No fenestrations were found in samples at the thoracic level. At present, we cannot determine the significance of these findings. We speculate that the presence of fenestrations at the lumbar spinal level may facilitate the transit of local anesthetics across the pia mater, possibly affecting the onset time or latency of subarachnoid blocks.

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