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The ependymal surface of the fourth ventricle of the rat: a combined scanning and transmission electron microscopic study

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Summary. The morphological features of the ependymal surface and supraependymal elements of the fourth ventricle of the rat were examined by scanning electron microscopy (SEM) and by the transmission electron microscopy (TEM). The results confirm the following aspects: 1) The presence of supraependymal elements and microvilli in the ependymal territories, including the sites where the cilia completely cover the ependymal surface; 2) The existence of cilia with oval or spherical thickenings together with supraependymal bulbs similar in size to those of the larger ciliary swellings; 3) Identification of the long supraependymal fibres with intermittent fusiform dilations observed under the SEM with the nerve fibres seen under the TEM; 4) The existence of intraventricular axodendritic synapses.

Key words: Rat, Ependyma, Fourth ventricle, SEM, TEM

Introduction

From the very start of the research on the ependymal layer of the cerebral ventricles, using the light microscope it has been possible to demonstrate: 1) the presence of several cellular types in this layer; 2) the existence of zones of different ciliary density; 3) the presence of neuronal bodies and of nerve prolongations contacting with the cerebrospinal fluid (CSF); and 4) the existence of cells inside the ventricles. These findings were corroborated, especially in the case of the third ventricle, by transmission electron microscopy (Leonhardt, 1966; Clementi and Marini, 1972; Peters et al., 1976) and by scanning electron microscopy. This latter technique is of particular use for the study of the supraependymal elements (Scott et al., 1973; Coates, 1973; Leslie et al., 1978) and also because of the possibility of examining large ventricular areas.

Whereas the ependymal surface of the third ventricle has been the object of much research using both the transmission (TEM) and scanning (SEM) electron microscopes, studies on the ependyma of the fourth ventricle with dense observational methods are very scarce. The work carried out on the ultrastructure of the ependymal surface of the fourth ventricle has essentially been reported in the form of reviews including this jointly with the whole of the encephalic ventricular epithelium (Fleischhauer, 1972; Mitro and Palkovits, 1981). Specific zones of the fourth ventricle have been studied both with TEM and SEM; an example is the area postrema, considered to be its circumventricular organ (Hofer, 1958). In this sense, the area postrema has been studied in birds (Hirunagi and Yasuda, 1979), in rodents (Torack and Finke, 1971) and in other mammals, including man (Scott et al., 1973; Klara and Brizze, 1977). There are also SEM studies of some regions of the floor of the fourth ventricle of the rabbit (Leonhardt, 1967; Leonhardt and Lindemann, 1973; Lindemann and Leonhardt, 1973), rat (Stumpf and Barbero, 1978; Collins, 1989), mouse (Yamadori and Yagishashi, 1975) and monkey (Singh et al., 1982) and of human embryos and fetuses (Scott et al., 1973). Some of these authors have carried out studies on the floor of the fourth ventricle combining transmission and scanning electron microsopy (Singh et al., 1982). The few studies of the roof of the fourth ventricle with SEM have been carried out in amphibians (Jones, 1979). In mammals, only the posterior part of this roof has been studied in the mouse (Oda and Nakanishi, 1987).

In previous works the ependyma both of the roof and of the floor of the rat fourth ventricle with SEM have been studied by us (Alvarez-Morujo et al., 1990a,b), demonstrating the existence of important

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variations in the distribution of the cilia and the microvilli over the surface of the ependyma. We also reported the presence of intraventricular elements near this surface, on which they are seen to lie as polymorphous protrusions, beaded supraependymal fibres resembling axons and different types of supraependymal cells (Alvarez-Morujo et al., 1986, 1989a,b).

In the present work, our aim was to carry a comparative study on the morphological features of the surface of the fourth ventricle obtained with SEM and with TEM.

Materials and methods

In the present study 12 adult Sprague-Dawley albino rats of 2 - 3 months of age were used, 6 of each sex. The animals were stabled with a light/dark regime of 12 h for each phase and temperature of 20° ± 2° C, relative air humidity 68 ± 5%, and a balanced diet and water *ad libitum*.

Before sacrifice, the rats were anaesthetized by intraperitoneal administration of a single dose of sodium thiopental (30 mg/kg b.w.). Thoracotomy was performed and the left cardiac ventricle cannulated. Later, under controlled pressure, the animals were perfused with heparinized physiological serum at body temperature. After cleaning the vascular tree, 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, 4° C, was injected through this. After completing the fixing perfusion, the brains were removed and with a frontal cut across the inferior colliculi the brain stem and cerebellum were separated from the rest of the encephalon. Of the 12 pieces comprising the brain stem and cerebellum, six (from three males and three females) were processed for SEM and the other six (also from three males and three females) for TEM.

The pieces for study with the SEM were immersed in the same fixative solution for 12 h at 4° C. After fixing, the roof and the floor of the fourth ventricle were separated by cutting the cerebellar peduncles, the choroid tela and the superior medullary velum. By two transverse cuts, three portions were obtained from both the roof and the floor: caudal, intermediate and cranial. These portions were postfixed in 1% osmium tetroxide in the same phosphate buffer, dehydrated in an ascending acetone series, dried by the critical point method and finally coated with gold. All the samples thus obtained were examined with a Philips PSEM-500 scanning electron microscope.

The pieces used for the TEM studies were immersed in a fixative solution for 12 h at 4° C. They were then washed with 0.1 M phosphate buffer at pH 7.4 with 6.84% sucrose and 0.01% $CaCl_2$ in two steps lasting 30 min each. The pieces were split with three frontal cuts, thus obtaining four portions, each of which in turn was cut sagittally. The eight resulting blocks were post-fixed in 1% osmium tetroxide in the same phosphate buffer, dehydrated as usual with an ascending acetones series and finally embedded in araldite. In the embedding step, attempts were made to place the block in the araldite in such a way that those from the left side would be cut on the frontal planes and those of the right side on sagittal planes. Using an LKB Ultrotome III 8000, semithin control sections were obtained from each of the blocks and stained with toludine blue. Ultrathin sections from 40 to 60 nm were also obtained and contrasted with lead citrate for observation under a Philips EM-201 transmission electron microscope.

Results

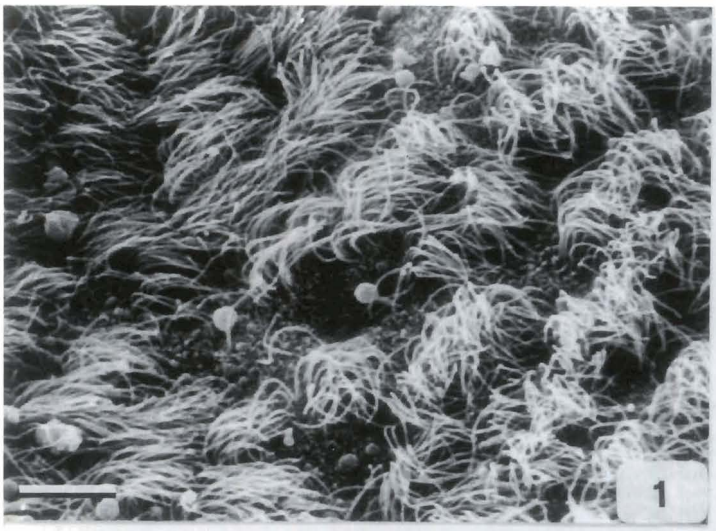
SEM examination of the floor and roof of the fourth ventricle revealed zones very rich in cilia accompanied by territories with fewer cilia, as shown in figure 1. In this figure, corresponding to a zone situated on the caudal third of the floor of the fourth ventricle, it is possible to observe numerous ciliary patches limiting small areas with no cilia and featuring the presence of spherical bulbs. Other territories were almost completely lacking in the cilia such as that shown in figure 2, which corresponds to a territory of the floor of the fourth ventricle.

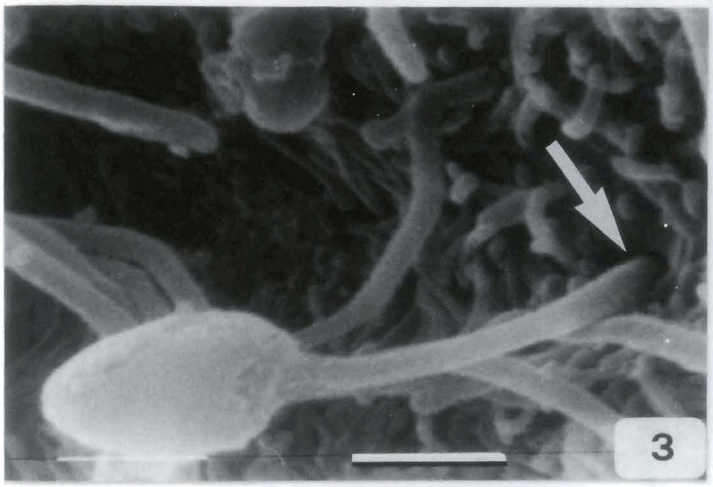
In the territories lacking in cilia microvilli of different sizes and shapes could be seen covering the apical pole of the ependymocytes; the density of the microvilli varied from one ependymocyte to another (Fig. 2).

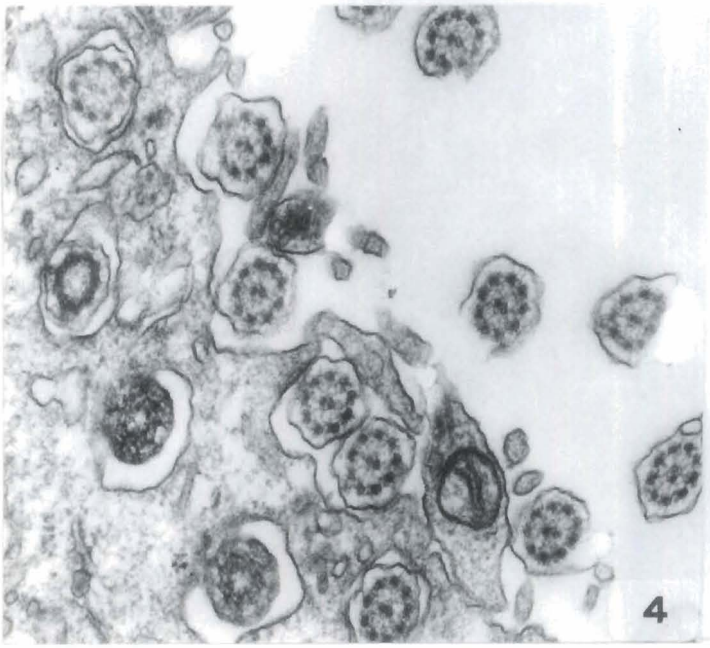
In these territories very long supraependymal fibres were seen that ran across the ventricular surface and showed spindle-shaped enlargements conferring them a bead-like aspect; often these fibres converged at a point, and some of them contacted with others at the point of convergence (lower part of Fig. 2). The calibre of these supraependymal fibres in the fourth ventricle of the rat ranged from 0.4 to 0.5 μm; the intercalated thickening measured approximately 1.5 μm in width and were from 4 to 5 μm long.

The territories of the fourth ventricle lacking in cilia could also exhibit small spherical or ellipsoid formations lying freely on the ependymal surface (Figs. 1, 2); these structures comprising a stalk emerging from the apical pole of the ependymocytes, which joined it to its dilated end. An interesting aspect of figure 3 is one of the latter structures. This is a stalk of some 3 μm in length and 0.2 μm in width that ends in a oval dilation; this ending has a length of approximately 2 μm (1.8 μm) and is about 0.9 μm wide. This structure is seen to project from the free surface of the ependymocyte towards the ventricular lumen, like the neighbouring cilia. Its width (0.2 μm) and lenght (almost 5 μm) correspond to the dimensions of a cilium. These structures in the shape of a stalk with terminal dilations appeared in different territories of the fourth ventricle, both on the floor and on the roof.

The TEM study of the ependymal surface of the fourth ventricle revealed, in some regions, an abundance of cilia (Figs. 4, 5). A transverse section of







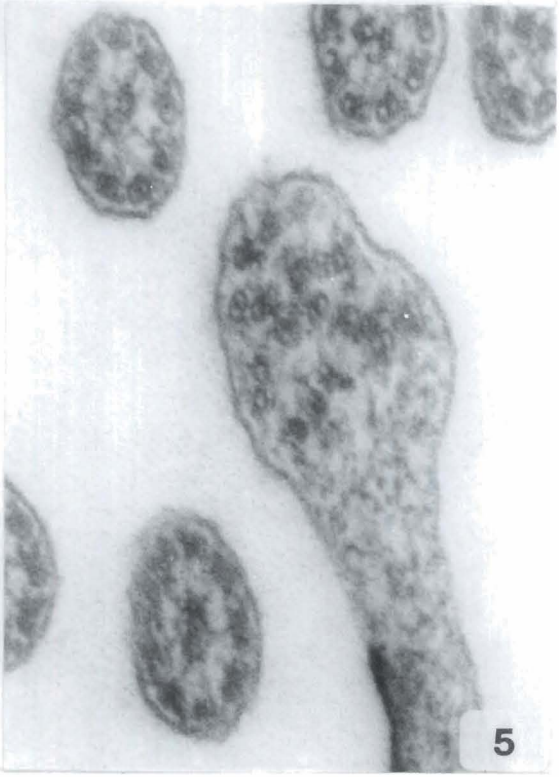


Fig. 1. Zone of the floor of the fourth ventricle of a female rat. Note the abundant patches of cilia, among which there are territories in which microvilli and apical blebs can also be visualized. (Bar = 10 μm).

Fig. 2. Territory without cilia of the ependymal surface of the floor of the fourth ventricle of a male rat. Of interest in this picture is the presence of supraependymal fibres with fusiform varicosities. Microvilli and spherical bulbs can also be seen (arrow). These latter are joined to the apical surface by stalks with a calibre similar to that of the cilia. (Bar = 5 μm).

Fig. 3. Roof of the fourth cerebral ventricle of a male rat. High power SEM image of a structure with a tail 0.2 μm wide ending in an oval enlargement about 2 μm long and 0.9 μm wide. Note that the stalk of this structure emerges from the apical pole of an ependymocyte (arrow). Cilia can also be seen (Bar = 1 μm).

Fig. 4. Apical surface of a ciliated ependymal cell from the floor of the fourth cerebral ventricle of a male rat. × 45,000

Fig. 5. Sectioned ciliary stalks with the classic 9 + 2 microtuble pattern from the roof of the fourth cerebral ventricle of a male rat. Note a stalk cut longitudinally ending in a dilated terminal in which there are microtubules grouped in doublets. × 103,000

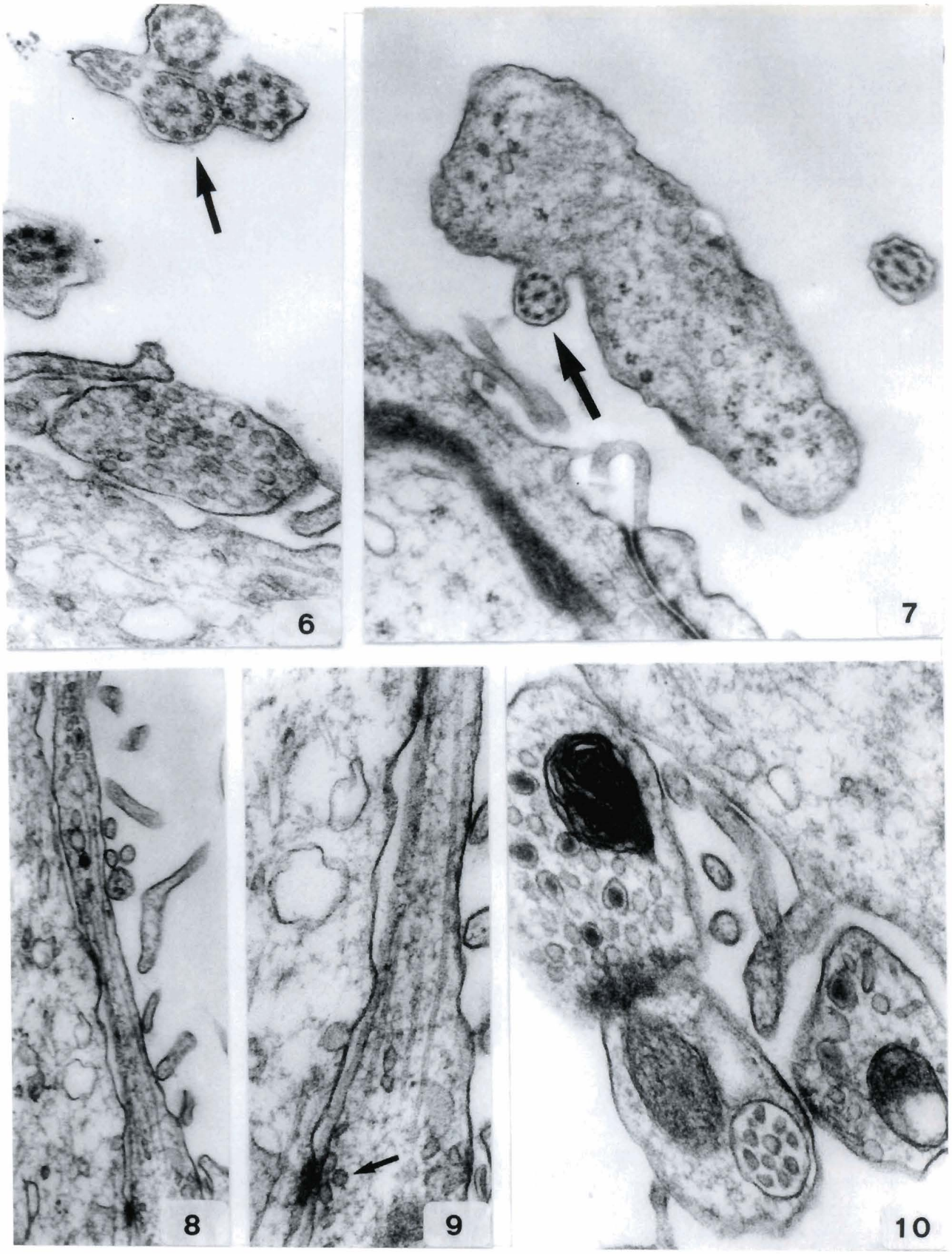


Fig. 6. Floor of the fourth cerebral ventricle of a female rat. Note several ciliary stalks encompassed within a single membrane (arrow). Above the apical pole of an ependymocyte there is a presynaptic nerve terminal. × 69,500

Fig. 7. Roof of the fourth cerebral ventricle of a female rat. Close to the ventricular surface there is a cytoplasmic mass containing a ciliary stalk (arrow). The membrane of the cilium continuous with that of the cytoplasmic mass. = 35,000

Fig. 8. Floor of the fourth cerebral ventricle of a male rat. A supraependymal fibre containing dense core vesicles, synaptic vesicles and microtubules can be seen. × 37,987

Fig. 9. Higher magnification (× 69,500) of the supraependymal fib re of the previous figure. The arrow shows a synapse *en pasant.*

Fig. 10. Floor of the fourth cerebral ventricle of a male rat. Note the axodendritic synapse in the fourth cerebral ventricle. × 69,500

the ciliary stalk showed its typical ultrastructural configuration, composed of 9 pairs of peripheral microtubules plus the two central microtubules (9 + 2 pattern). These cilia were intermingled with microvilli of different sizes (Fig. 4). It should be noted that these microvilli were intimately related to the cilia and to other bulbous structure containing large numbers of small vesicles that could be identified as terminal nerve boutons (Fig. 6).

In figure 5 a longitudinal stalk, of smaller calibre than that of the cilia, with a dilated end is seen; the dilation displays microtubules, which form doublets arranged irregularly instead of in circular shape. The presence of the microtubule doublets suggests that this formation would be a cilium with a dilated end.

It is sometimes possible to observe several ciliary stalks with the typical internal structure (9 + 2 pattern) grouped with a common membrane (Fig. 6). Close to the ependymal surface of the floor of the fourth ventricle, a flattened cytoplasmic mass was found. The major axis of this measured approximately 2 μm (Fig. 7). On the side facing the ependyma, the cytoplasmic mass exhibited a ciliary stalk, with its characteristic tubular content; the membrane of the ciliary stalk coursed continously with the cytoplasmic mass, indicating that they form a unity.

The TEM studies also revealed the presence of intraventricular nerve fibres lying on the apical membrane of the ependymocytes. When cut longitudinally these fibres showed a homogeneous calibre along most of their course with some dilations in their trajectory (Figs. 8, 9). Inside, the fibre displayed the specific organelles of a nerve fibre, such as microtubules, several vesicles with an electron-lucent content, few dense core vesicles, and some mitochondria. There was a point on this fibre, very close to the apical membrane of the ependymocyte, at which there were several synaptic vesicles; at this level, the membrane of the apical pole of the ependymocyte exhibited a stronger osmiophilic density; the whole resembling synapses *en passant* (Fig. 9).

Within the ventricular space synapses were observed, like those depicted in figure 10. One of the elements of this synapses was a terminal nerve bouton containing a myelin-like figure, dense core vesicles, and other synaptic vesicles. The other element may be an intraventricular dendrite in which the presence of a multivesicular body was striking. In our opinion, this would be an axodendritic synapse.

Discussion

The combined SEM and TEM study reported in the present work contributes to a better knowledge of the ependymal surface of the rat brain fourth ventricle. Our examination of the ependymal surface of this ventricle with SEM confirms the existence of three types of zones with respect to the number of cilia contained in them: 1) zones in which the cilia are so abundant that they cover the whole of the ventricular surface; 2) zones with no cilia; 3) zones that, although very rich in cilia, exhibit territories with no cilia (Alvarez-Morujo et al., 1990b). On the floor of the fourth ventricle Singh et al. (1980) have also described regional variations in the form of dense ciliation, sparse ciliation, central tufts of cilia, solitary cilia, etc.

Additionally, in sites of the ependyma where SEM showed the cilia completely hiding the ventricular surface, TEM permitted one to visualize this surface. In these sites there were microvilli and supraependymal nerve elements; specially fibres and nerve boutons underlying the cilia. The irregularly-distributed microvilli of different sizes and shapes suggest that where they are present the ependyma might carry out functions of absorption and reabsorption (Santolaya and Rodríguez-Echadia, 1968; Koshiba, 1987).

With SEM, stalks were observed emerging from the ependyma; some of these exhibited ellipsoid or round thickenings on their distal intraventricular terminations. These structures may be cilia with cytoplasmatic masses joined to their stalks like those observed with TEM. Singh et al. (1980) have observed the existence of preterminal bulbous enlargements in some cilia of the floor of the fourth ventricle of the rat, although these authors only offer SEM images, as have Yamadori and Yagihashi (1975) who carried out their studies in mice. Mitro and Palkovits (1981) have reported TEM images of cilia with small cytoplasmic dilations in the third ventricle of the rat. These authors state that «occasionally we found cilia with cytoplasmic thickenings that contained vacuoles varying in size». Thus, electron microscopy confirms the existence of cilia with dilated ends in the fourth ventricle of the rat. With SEM we have demonstrated that these cilia with distal thickenings are present in different territories both of the floor and the roof of the fourth ventricle; although they are not very numerous, their finding is not exceptional. Such ciliary thickenings might represent a receptor specialization of the ependymocytes. This suggestion is based on the transformation of some cilia into receptor structures, such as the olfactory cilia or those of other cell types from lower animal species (Golding and Whittle, 1977; Oksche, 1985, 1988). Moreover, in a study on differentiation of the embryonic chick pineal organ, Möller and Möller (1990) reported that the sensory apparatus of the pinealocytes «is achieved by development of an oval-shaped, biconcave swelling at the tip of the cilium, 1 x 2 μm in size».

Inside the rat brain fourth ventricle, together with these modified cilia, there are supraependymal bulbs, similar in size to those of the larger ciliary swellings. Apparently, these formations are independent of the ependymocytes and lie freely on the ventricular surface. The detachment of the distal thickening might be the result of the dynamic capacities of the intraciliary microtubules (Schwartz, 1980). If this were the case, the cilia with dilated ends might be the expression of a particular secretory mechanism of

cytoplasmic substances from the ependymocyte to the CSF. This emphasizes the sensory and secretory capacity displayed by some ependymal cells of lower vertebrates (Golding and Whittle, 1977; Oksche, 1985).

The spherical or ellipsoid supraependymal bulbs have been interpreted as the expression of an apocrine process in the lateral ventricle of the rabbit brain (Hetzel, 1978) and in the third ventricle of the rat brain (Pastor et al., 1981); other authors, however, have postulated that they might only be artefacts (Ribas, 1977). Mestres et al. (1985) have also suggested that the supraependymal bulbs «may also appear as the result of a final vital reaction if fixation is delayed by a protracted period of vascular rinsing prior to perfusion of the fixative».

In the ependyma of the rabbit brain fourth ventricle, Leonhardt (1967) have found large knoblike protrusions containing numerous mitochondria; these structures were first reported by Takeichi (1966) in the kitten, although this author did not provide any information about the number of location of these bulbs (Fleischhauer, 1972).

According to Leonhardt (1967), Leonhardt and Lindemann (1973) and Lindemann and Leonhardt (1973), the *mitochondria-containing bulbs* are «end-organs of subependymal cells and have fingerlike protrusions, but no synapses and no sensory cells» (Leonhardt and Linderman, 1973); they are indicative of an intraventricular neurosecretion or «may be receptors», but their function is not yet known for certain. In the central canal of the cat, Rascher et al. (1985) also found numerous *mitochondria-containing bulbs*, which in some respects resemble the processes of CSF-containing neurons.

In our work we found no TEM images corresponding to these *mitochondria-containing bulbs*, which, on the other hand, does not exclude their presence in the fourth ventricle of the rat.

Among the supraependymal elements observed by us with the SEM in the fourth ventricle, both in the present work and in previous ones (Alvarez-Morujo et al., 1986, 1990a,b), outstanding is a network of long fibres with a bead-like aspect evidenced by intermittent fusiform dilations. In the preparations analyzed with the TEM, both from the floor and the roof of the fourth ventricle, we have also observed supraependymal fibres cut longitudinally, inside which there are organelles typical of nerve fibres: microtubules, dense core vesicles, light vesicles, etc. The calibre of the fibres and the presence of thickenings allows one to identify the nerve fibres visualized with the TEM with the bead-like fibres observed with the SEM. In other words, the supraependymal fibres with a bead-like aspect of the fourth ventricle of the rat observed with SEM are in fact nerve fibres and probably form part of the supraependymal nerve plexus described in depth by several authors (Leonhardt, 1967, 1968; Leonhardt and Backhus-Roth, 1969; Leonhardt and Linderman, 1973; Linderman and Leonhardt, 1973, etc). This supraependymal nerve plexus was described by Derer (1981) using the Golgi method in several ventricles of the mouse brain.

In the preparations analyzed with the SEM we observed (Alvarez-Morujo et al., 1990a) that some dilated ends of the supraependymal fibres join others, to form sets resembling synapses. SEM, however, does not allow one to confirm the existence of such synapses. Accordingly, do intraventricular synapses exist? TEM confirms their existence, as depicted in figure 10 of this work, showing a synapse that we interpret as an axodendritic synapse in the lumen of the fourth ventricle of the rat. Previously, Scott et al. (1975) obtained electron micrographs of terminal nerve boutons on the soma of supraependymal neurons (axomatic synapse) on the floor of the third ventricle of normal male rats.

The exact function of the supraependymal elements and of their origin remains to be elucidated. It is believed that: 1) the supraependymal neurons would be involved in the reception of physico-chemical changes in the CSF; 2) they may produce and release bioactive substances into the CSF; and 3) they regulate the function of the CSF-contacting neurons. Richard et al. (1973) suggest that the intraventricular fibres are serotoninergic and that they would originate in the raphe nuclei of the brain stem. Using the SEM, we have observed (Alvarez-Morujo et al., 1987) supraependymal fibres similar to those with a bead-like aspect entering the ventricular surface, as though we were dealing with a cilium. Probably, these fibres either course towards other territories of the nervous system or arise in them.

Is there any correlation between a given pattern of the ependymal surface and a specific type of the underlying nervous system? In a study of the lateral ventricle of the pigeon brain with SEM Mestres et al. (1985) suggest that such a correlation does not exist. In our experience with the infundibular recess of the third ventricle (Amat, 1989) this correlation is evident, especially at the level of the arquate nucleus of the hypothalamus. Indeed, the ependymal pattern of this territory is characterized by the presence of tanycytes and of flattened ependymocytes lacking in cilia and by the absence of the glial subependymal layer. The absence of this layer suggests that the arquate nucleus might receive more direct influences from the CSF (Amat et al., 1991).

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