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ULTRASTRUCTURAL STUDY OF MYELINATING CELLS AND SUB-PIAL ASTROCYTES IN DEVELOPING RAT SPINAL CORD

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SUMMARY

The anterior funiculus of the spinal cervical cord of post-natal rats was examined ultrastructurally. The myelinating cells found one day after birth contained a large amount of evenly distributed ribosomes up to the outer tongue of mesaxons, representing the cytoplasmic density. These cells were separated by astrocytic processes from the pial basement membrane, even when they were located on the pial surface. Astrocytes contained glial fibrils from one day onwards and often attached their processes to the pial basement membrane. Although the cytoplasmic processes of astrocytes occasionally wrapped axons, they were never shown to form the initial layer of myelin sheaths. However, the tenuous processes of the sub-pial astrocytes were occasionally rolled in myelin lamellae, as if a part of the myelin sheaths was constructed by astrocytic processes. The interpretation for this finding is discussed in relation to function and potency of the astrocytes, and variations and anomalies of nervous ontogeny.

INTRODUCTION

Myelinogenesis in animals during postnatal development has been extensively examined by several investigators. They examined myelinogenesis in the optic nerves of rats or rabbits (Vaughn 1969; Skoff et al. 1976; Tennekoon et al. 1977; Narang 1977), in the cerebrum of cats, rats or mice (Bunge et al. 1962; Kruger and Maxwell 1966; Fleischhauer and Wartenberg 1967; Caley and Maxwell 1968; Froher 1976;

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Kozik 1976) and in the spinal cord of rats (Matthews and Duncan 1971; Knobler et al. 1974, 1976). Light- and electron-microscopic studies have demonstrated that oligodendrocytes are involved in myelinogenesis in the normal central nervous system (see review of Bunge 1968), although in the past a concept was proposed that myelin is constructed as a patchwork assembly of many different glial processes (Luse 1956; Ross et al. 1962). This viewpoint was supported by Narang (1977), who suggested in his study of the rabbit optic nerve that astrocytes also myelinate axons. Moreover, in an investigation of virus-induced demyelination in rats it was shown that astrocytes as well as oligodendrocytes undergo morphological changes prior to demyelination, suggesting an interrelationship of myelin with these two cell classes (Nagashima et al. 1978). This observation, together with Narang's findings led to the present examination of the relationship between glial cells and myelin sheaths in the developmental stage in relation to the role of astrocytes in myelination in areas other than optic nerve. As a site of myelinogenesis the anterior funiculus of the spinal cervical cord of rats was chosen since it was found to be the preferred area of corona virus-induced demyelination in our animal model.

MATERIAL AND METHODS

Normal pregnant rats, strain CHBB/THOM, were purchased from Thomae, Biberach, F.R.G. and used for the experiments. The animals were perfused by direct cannulation into the hearts on 1, 3, 5, 9 and 21 days after birth. Two animals were examined at each time. The perfusate consisted of 2.0% glutaraldehyde in 0.1 M phosphate buffer solution pH 7.4 at room temperature. After perfusion the spines with spinal cord were dissected and they were cut horizontally through intervertebral discs. The divided blocks of the spinal cords which correspond to the 2nd and 4th cervical vertebral bodies were fixed overnight in the same fixative as the perfusate. After fixation the spinal cords were removed carefully from the spines and sliced horizontally into 1-mm thick sections. The sections were post-fixed in 1% osmic acid, stained en bloc with 2% uranyl acetate in 0.1 M sodium hydrogen maleate buffer (Karnowsky 1967), dehydrated and embedded in epoxy resin. Sections were prepared from the horizontally cut surfaces. Semi-thin sections were stained with 1% toluidine blue. The ultra-thin sections from the selected areas were stained with lead citrate and examined with a Zeiss 10 B electron microscope.

Terminology

To avoid terminological misinterpretations the terms "wrap" or "enfold", and "ensheath" or "myelinate" were used differently: "wrap" or "enfold" were applied when the glial processes hold axons, but the fusion of their cytoplasm to form a compact group of myelin lamellae was not found. The terms "ensheath" or "myelinate" were employed when the cytoplasm surrounding the axons coalesced at least in some parts to form either an intraperiod line or a major dense line (Caley and Maxwell 1968; Knobler et al. 1976) or when the layers of myelin lamellae had been constructed. The cells whose cytoplasmic processes were found to be continuous with the

ensheathing or myelinating spiral were referred to as myelinating cells. The cells attaching their cytoplasmic processes to the basement membrane of blood vessels or pia mater were referred to as astrocytes, containing, in addition, glial fibrils with a diameter of 70–80 Å.

RESULTS

Histological findings

One- μm toluidine blue-stained sections of the spinal cervical cord of 1-day-old rats showed several myelinated axons in the anterior and posterior and none in the lateral funiculus. In the marginal layer of the anterior funiculus low triangular cells with wide cytoplasmic processes were observed. Some of their cytoplasmic processes contained myelinated axons (Fig. 1a). The size and number of these cells increased with age. A few cells located on the pial surface also contained myelinated axons (Fig. 1a). The cells located in the matrix attached one process to the pial surface and the other to myelinated axons (Fig. 1b). From the 3rd day onwards myelinated axons were present throughout the spinal white matter and on the 5th day many axons in the anterior funiculus were myelinated. Few capillaries were found in this area. From the 5th day onward the number of cells and myelinated axons rapidly increased and cytoplasmic contacts with the myelin sheaths were difficult to find.

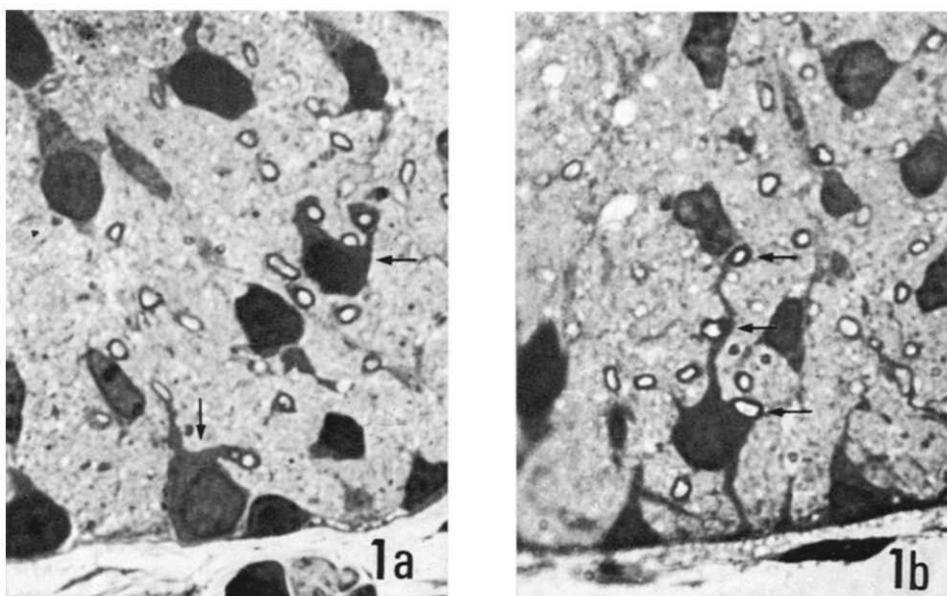


Fig. 1. Anterior funiculus of a 1-day-old rat. Toluidine blue staining, $\times 900$. *a*: a few axons are being myelinated. In the center two myelinated axons are surrounded by the cytoplasmic processes of one cell (\leftarrow). Another cell (\downarrow), at the left bottom, extends two processes to the myelinated axons. This cell seems to be located in contiguity with the pial basement membrane on this specimen, but electron microscopy revealed that the thin astrocytic processes were interposed between this cell and the pial basement membrane, as shown in Fig. 3. *b*: a cell extending two long processes. One to the pial surface and the other into the matrix. Three myelin sheaths are found to be connected with this cell (\leftarrow).

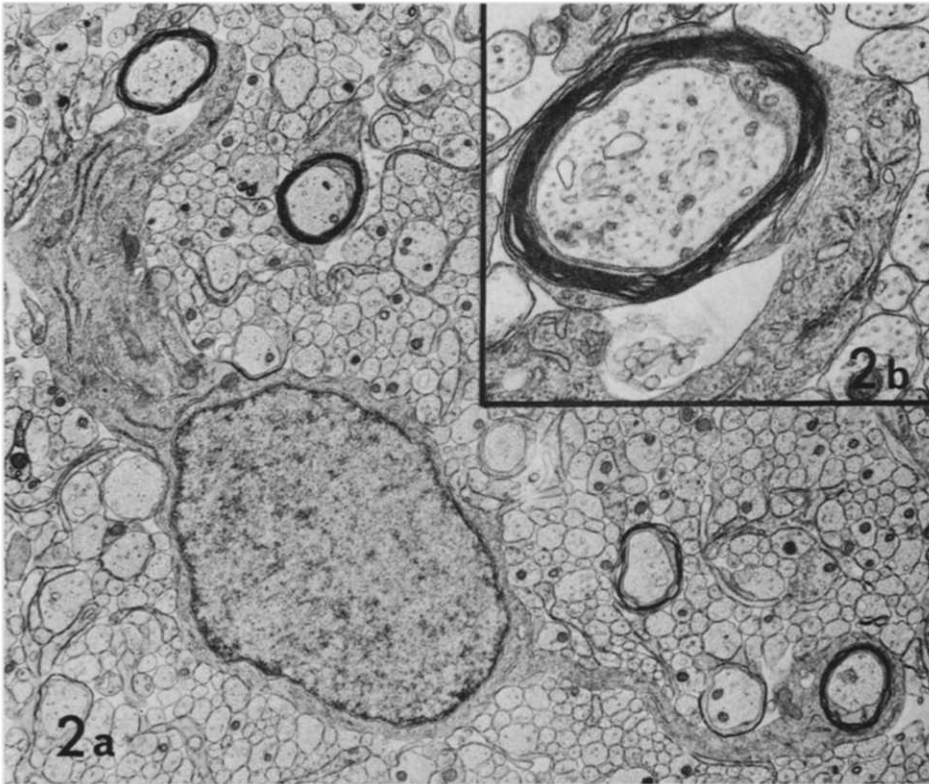


Fig. 2. Myelinating cell of a 1-day-old rat. *a*: the cytoplasmic processes are found continuing to the two myelin lamellae (left upper corner and right lower corner). The cytoplasm contains the cisternae of granular endoplasmic reticulum, Golgi apparatus and mitochondria. Note the homogeneous density of the cytoplasm, and the fact that larger axons are well myelinated. $\times 7800$. *b*: higher magnification of the myelinating process of the left upper lamella in *a*. Note the presence of vesicular structures and evenly distributed free ribosomes in the process.

Electron-microscopical findings

Ultrastructurally the large cells in the matrix of 1-day-old animals were confirmed to be myelinating cells with cytoplasmic processes reaching the outer mesaxons of the myelin spirals (Fig. 2a). The cytoplasm of these cells contained a large amount of evenly distributed free ribosomes up to the mesaxons (Fig. 2b). In addition to the usual organelles, such as mitochondria and Golgi apparatus, these cells contained cisternae of granular endoplasmic reticulum, small vesicles and microtubules, but no glial fibrils. The cells located on the pial surface, containing the myelinated axons in their cytoplasm (Fig. 1a), were proved to be the myelinating cells by electron-microscopical examinations (Fig. 3). The cytoplasmic features of these cells were identical with those of myelinating cells in the matrix. On histological examination these cells appeared to be lining up to the pial surface. Electron microscopy, however, revealed thin cytoplasmic processes separating the myelinating cells from the pial basement membrane (Fig. 3).

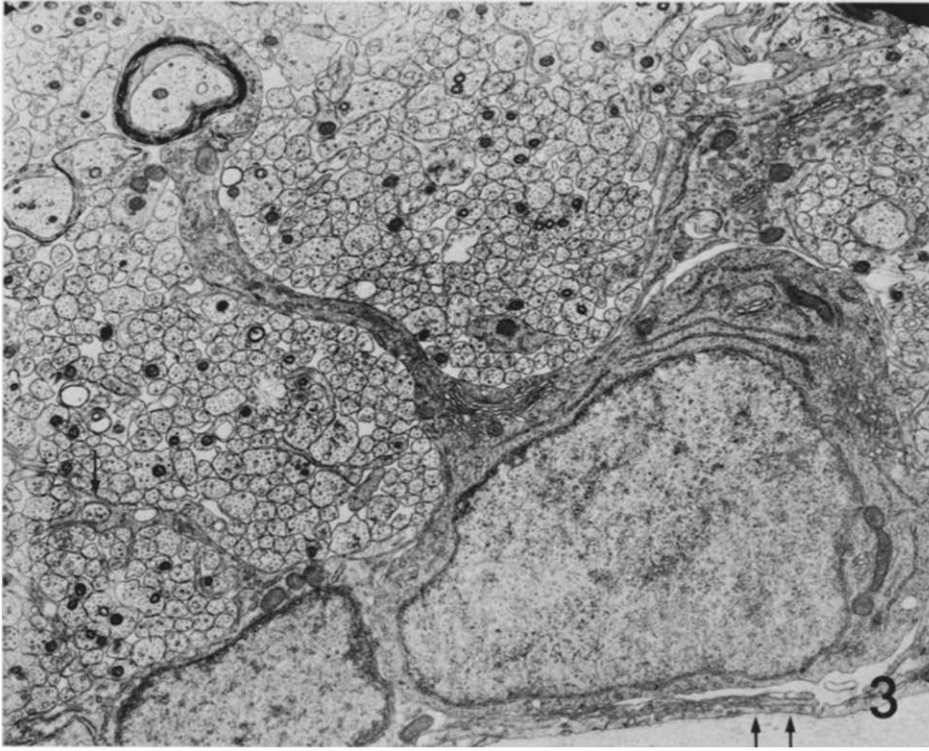


Fig. 3. A myelinating cell located on the pial surface as found in Fig. 1a. A long and wide process extends to myelinate the largest axon of this field (left upper corner). The thin cytoplasmic processes that are penetrating in order to separate them are found between this cell and the pial basement membrane ($\uparrow\uparrow$). An astrocyte to the left of the myelinating cell extends its cytoplasmic processes. Note that one of these processes is wrapping itself around a small axon (\downarrow). $\times 10,000$.

Therefore, these myelinating cells neither contained glial fibrils nor showed direct contact with the basement membrane, lacking features pertaining to the astrocytes.

Other cells which were contiguous with the pial basement membrane exhibited glial fibrils, mitochondria, Golgi apparatus, vesicles and free ribosomes even in a 1-day-old rat (Fig. 4). These cells were considered astrocytes and their ribosomes were not as homogeneously distributed as found in the myelinating cells. This low density of the cytoplasm could be traced to the tip of the cytoplasmic processes (Fig. 4). The cytoplasmic processes of the astrocytes often wrapped the naked axons (Figs. 3 and 4). In these wrappings the processes neither fused their inner aspect to form a major dense line, nor revealed the coalescence between their outer and inner layers forming an intraperiod line as shown in the initial myelinating layer of axon (A2) of Fig. 4. Moreover, the cytoplasmic processes enfolding axons contained very few ribosomes or vesicles, differentiating them from those of the myelinating cytoplasmic processes. The astrocytic processes also contained, besides glial fibrils, a large number of microtubules. The presence of microtubules was confirmed up to the 21st day, although the

number decreased with age of the animals. The most interesting phenomenon was the enrolment of astrocytic processes by myelin lamellae (Figs. 5 and 6). This phenomenon was occasionally observed in the sub-pial astrocytes. The tenuous astrocytic processes trapped in the lamellae did not obviously fuse or coalesce their cytoplasmic membrane to form the myelin lamellae. In Fig. 5b an attenuated astrocytic process could be traced clockwise through one and a half turns inside the lamellae with the same direction as the inner tip of the mesaxon and this process was seen to be overlapped by the counter-clockwise rotation of the outer tongue. In Fig. 6c, a penetrating astrocytic process was covered by one half turn of a major dense line which is continuous with the outer tongue of the mesaxon. These findings were observed until the rats became 5 days old; thereafter they were difficult to detect, probably because of the complexity of the developing central nervous system and the consequent difficulties in interpretation.

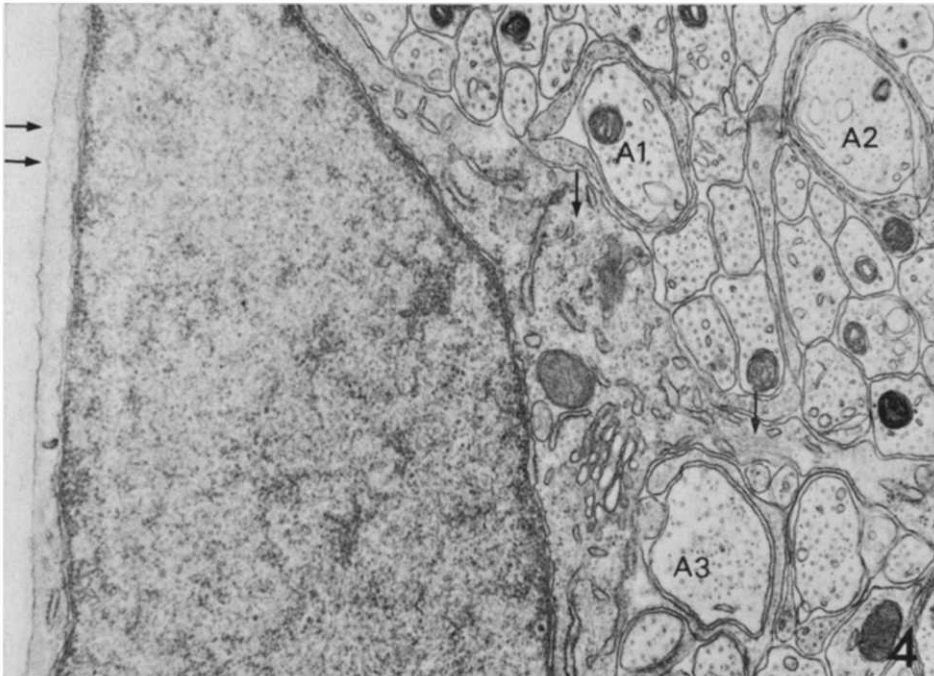


Fig. 4. A sub-pial astrocyte of a 1-day-old rat. The cytoplasm of astrocyte faces the pial basement membrane (left side, \rightleftharpoons). On the opposite side, it contains various organelles. The cytoplasmic density is not homogeneous, compared to those of the myelinating cells. In this field, two axons are wrapped (A1) or ensheathed (A2) by cytoplasmic processes containing evenly distributed electron-dense granules. Around an axon A2, an intraperiod line produced by the interconnection of the inner and outer mesaxons can be seen. While an astrocytic process wrapping itself around an axon (A3) shows less electron density and forms neither major dense line, nor intraperiod line. Note the presence of thin glial fibrils (\downarrow) in the cytoplasm of astrocyte. $\times 15,000$.

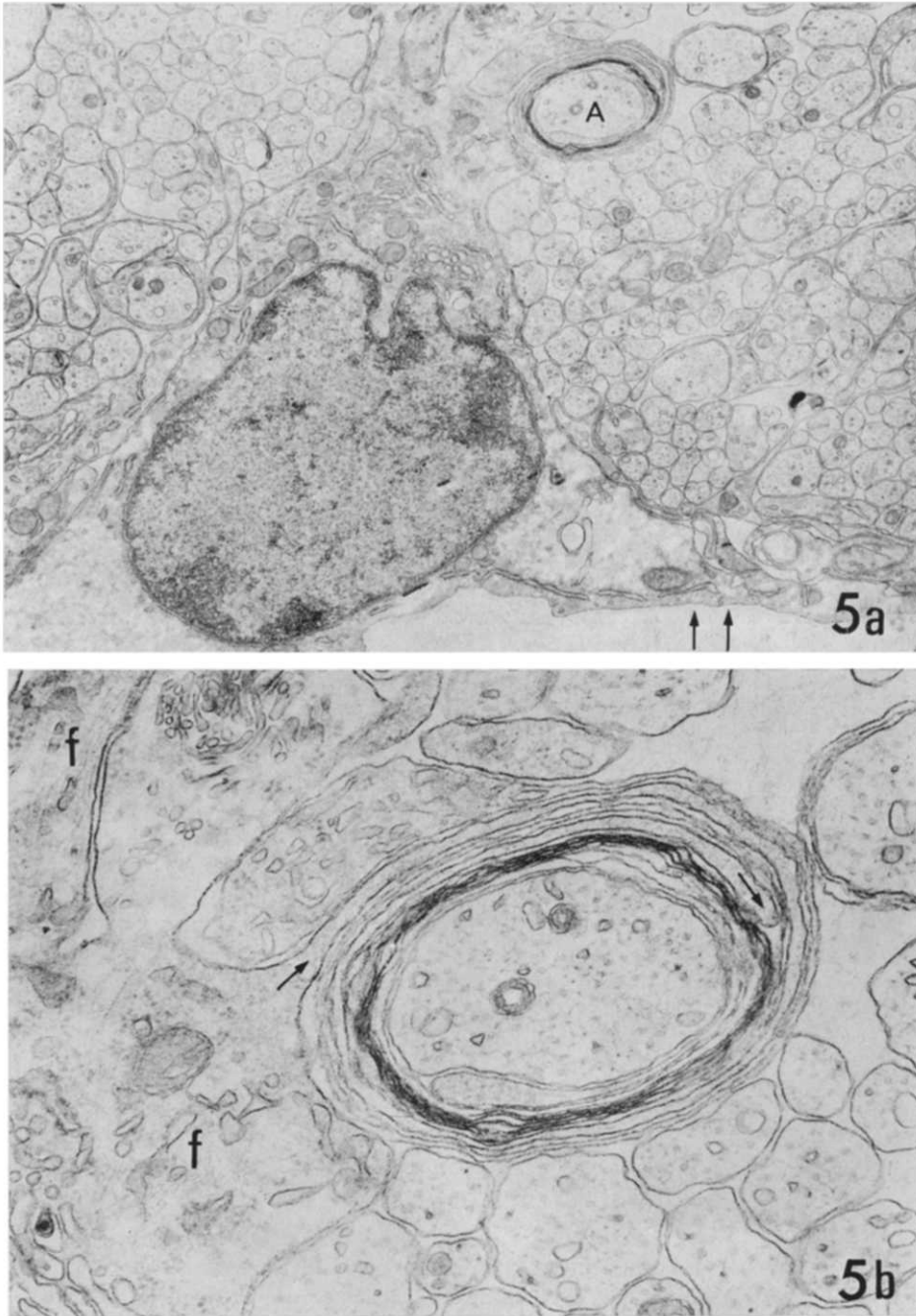


Fig. 5. A sub-pial astrocyte of a 1-day-old rat. *a*: an astrocyte located on the pial basement membrane ($\uparrow\uparrow$) extends its cytoplasmic process into the myelin lamellae of the largest axon (A) in the field. $\times 9000$. *b*: higher magnification of the axon (A) in *a*. The cytoplasmic process is wrapped clockwise one and a half turn around the myelin lamellae. The arrow (\nearrow) can be continuously traced to the other arrow (\curvearrowright). The outer tongue contains vesicular structures (ot). A small amount of glial fibrils is seen in the left area (f). $\times 33,000$.

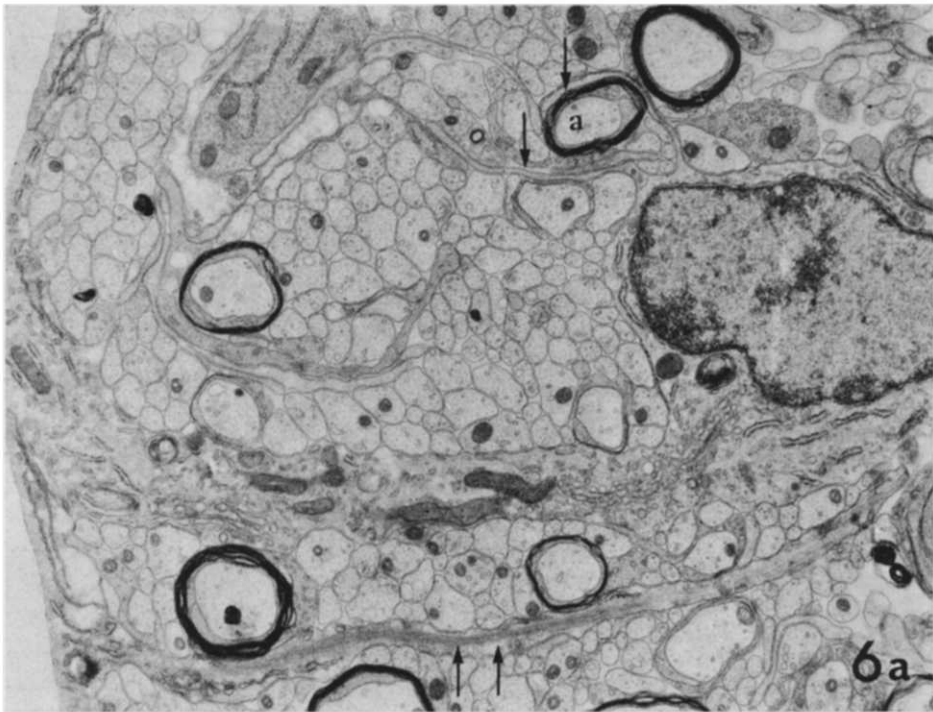


Fig. 6. An astrocyte of a 5-day-old rat. *a*: the cytoplasm containing various organelles attaches to the pial basement membrane in the left side. This cell extends two tenuous processes (\downarrow), and one of them around an axon (*a*) is encased inside of the myelin lamellae. At the bottom, another astrocytic process containing a long and compact bundle of glial fibrils can be seen ($\uparrow\uparrow$). Note that only the large axons are myelinated. $\times 16,000$. *b*: higher magnification of the pial part of the cell. In the center glial fibrils are seen (\leftarrow). $\searrow\searrow$: basement membrane. $\times 26,000$. *c*: higher magnification of the one process around an axon (*a*) in *a* shows that the outermost myelin lamella actually encases the astrocytic process. A cytoplasmic process (\rightarrow) can be traced to the tip (\rightarrow). Compared to the inner and outer tongues of mesaxons, the astrocytic process is lighter and lacks organelles. Note that the outermost lamellae consists of a major dense line ($\leftarrow\leftarrow$) made by the fusion of the inner aspect of the cytoplasmic membrane of the outer tongue (*ot*). $\times 26,000$.

DISCUSSION

It is generally accepted that astrocytes in the postnatal period attach their processes to the basement membrane of blood vessels or pia mater and contain glial fibrils. Myelinating cells lack these specific structures. Their cytoplasm is rendered denser by a homogeneously distributed larger number of free ribosomes. This feature of myelinating cells is identical to that of oligodendrocytes (Bunge 1962; Kruger and Maxwell 1966; Hirano, 1968; Vaughn 1969; Mori and Leblond 1970; Knobler 1976; Skoff et al. 1976; Tennekoon et al. 1977), supporting the general concept of myelinating axons by oligodendrocytes.

The possibility that astrocytes are involved in the process of myelination was already suggested by Wendell-Smith et al. (1966). In their study of cat optic nerves, astrocytes in the lamina cribrosa were thought to be responsible for the formation of the fine myelin sheaths, since no oligodendroglia are located in this region. They regarded the astrocytic wrappings around axons as the initial ensheathment. However, Vaughn (1969) in a reevaluation of these studies came to the conclusion that astrocytes are probably not involved in the early stage of myelination. His interpretations were based on the following findings: the observed wrappings extended only short distances along the length of the enclosed axons, typical mesaxons were never detected in such wrappings (Vaughn and Peters 1967) and a time difference was seen between the occurrence of wrappings and the initial myelin sheaths. He concluded that the occasional wrapping of unmyelinated axons by astrocytic processes would seem to be a random event associated with the outgrowth of astrocytic processes forming astroglial sheets rather than with the production of myelin sheaths. In the present study, the initial layer of ensheathment was not observed in such wrappings. Moreover, the cytoplasmic density in tips of wrappings was lighter than those of myelinating processes. Therefore, in agreement with Vaughn's conclusion, astrocytic wrappings were not considered the initial ensheathment.

Recently, Narang (1977) in his study of the epiretinal portion of post-natal rabbit optic nerve stated that astrocytes may myelinate axons by demonstrating myelinating cells with foot processes linking to blood vessels. However, the attachment of the foot processes to the blood vessels seems to be questionable, since in Narang's Fig. 6 other cytoplasmic processes interpose between the myelinating cells and the blood vessel, as was also found in this study (Fig. 3).

Although we could not prove that astrocytes myelinate axons, a peculiar configuration could be demonstrated. Astrocytic processes were actually trapped in the myelin lamellae, as if a part of the myelin sheaths were constructed by astrocytic processes (Fig. 5). There has been no reported demonstration that astrocytic processes were rolled in myelin lamellae. Luse (1956) in her study of mouse and rat brains and spinal cords suggested that many glial cell processes may be involved in myelin formation rather than a single cell and its processes. One could argue about the trapped process developing into a myelin component, because the direction of the astrocytic spiral is contrary to that of the outer tongue of the lamellae. Knobler et al. (1976) in their analysis of serial electron micrographs of the lumbar spinal cord white

matter of 5-day-old rats showed that the direction of early ensheathment spirals was often bidirectional. The same finding can be seen in Figs. 5b and 6c. The spiral direction of the inner tongue and astrocytic processes are clockwise, while that of the outer tongue is counter-clockwise.

It would be an additional argument against this type of astrocytic process becoming a myelin component that the process trapped in the myelin lamella never fused or coalesced their cytoplasmic membrane to form the myelin lamella. The enrolment of the astrocytic process by myelin lamellae was in our observation only recognized in subpial astrocytes. In the prenatal neuroglial development, the subpial glial cells or glioblasts are produced from matric cells by their migration to the periphery of the spinal cord and their transformation (Fujita 1965). These glioblasts mature into oligodendroglia and astrocytes, as development proceeds. And in neonatal and early post-natal ages, glial cells divide and proliferate actively (Sakla 1965; Gilmore 1971). In these stages, therefore, the subpial astrocytes may have potency to differentiate into both astrocytes and oligodendroglial cells.

It is well known that there often occur variations, anomalies and errors in neuronal ontogeny. Hildebrand (1971) showed in the developing feline spinal cord that myelination is accompanied by a disintegration of some sheaths and demyelination of short internodes, as well as degradation of some oligodendroglial cells. Fleischhauer and Wartenberg (1967) found in their studies of developing feline corpus callosum the bizarre evaginations of myelin sheaths, which correspond to the "redundant myelin" described in the brain of *Bufo* by Rosenbluth (1966). Taking these observations into account, the myelin lamellae containing astrocytic processes may be a developmental fault in myelinogenesis.

Another explanation for this phenomenon is that the enrolment of astrocytic processes is a transient event and they may be released again from the lamellae in the subsequent growth of myelin sheaths. This idea is based on the hypothesis that one of the astrocytic functions during development is to guide some neurons during their outward migrations from the germinal zone to their final resting positions (Jacobson 1978). Reier and Webster (1974) suggested in their studies of tadpole optic nerve remyelination that astrocytic processes guide oligodendrocytic processes as they extend to surround axons.

At the present time, it remains open to discussion whether the enrolled astrocytic process becomes a part of the myelin lamellae in the subsequent development of the central nervous system, or serves merely as a pilot for the development of myelin sheath.

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