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Initial development of capillaries in the neuroepithelium of the mouse

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INTRODUCTION

It has repeatedly been demonstrated that in addition to the differentiation of the neuroepithelial cells, the degree of vascularisation of the embryonic CNS also constitutes an important developmental step in its functional maturation (Feeney & Watterson, 1946; Lierse, 1963; Caley & Maxwell, 1970; Bär, 1980). This correlation between vascularisation and function serves to stimulate interest in the question of how the process of vascularisation of the embryonic CNS *anlage* begins.

In the CNS *anlage* of rat embryos, the first capillaries are found in the parietal–temporal region of the developing hemispheres (Bär, 1980) and in the developing spinal cord (Simon-Marin, Vilanove, Aquinagalde & Barbera-Guillem, 1983) between Day 12 and Day 13 of development. In the CNS *anlage* of mouse embryo these first capillaries can already be seen in the developing spinal cord on Day 10 of development (Sturrock, 1981). However, in teratological studies on the CNS *anlage* of 10 days old mouse embryos, we found numerous capillaries in the neuroepithelium of the prosencephalon and the developing spinal cord (Herken, Merker & Krowke, 1978; Herken, 1985), i.e. one must assume that vascularisation of the CNS *anlage* of the mouse embryo begins before Day 10 of development.

The aim of the present investigation was to pinpoint histologically the exact stage of development and the location within the neuroepithelium at which capillarisation of the CNS *anlage* of the mouse embryo begins. This investigation was carried out on 5–7 μm serial sections of 9 and 10 days old mouse embryos embedded in paraffin, as well as on 1 μm serial sections of Epon-embedded embryos. At the ultrastructural level, we investigated also the structural changes accompanying the initial stages of vascularisation.

MATERIAL AND METHODS

NMRI mice were kept on a normal day/night cycle and given Altromin commercial food and water *ad libitum*. The day on which, at 11 a.m., a vaginal plug was detected, after a mating period of 3 hours, was designated Day 0 of gestation. On Day 9, Day 9 plus 5 hours, Day 9 plus 10 hours, Day 9 plus 20 hours and Day 10 the pregnant animals were killed by cervical dislocation and the embryos were removed. For serial sectioning the embryos were fixed in Bouin's fixative and embedded in paraffin. From each stage of development, cross-sections were cut from 4 embryos and longitudinal sections from 2 embryos and stained with haematoxylin and eosin.

Embryos from Day 9 and Day 9 plus 5 were fixed in a mixture of 1% glutaraldehyde and 1% tannic acid in 0·1 M phosphate buffer, postfixed in 1% OsO_4 in 0·2 M phosphate buffer and embedded in Epon. From this material, 1 μm cross-sections

were cut from each of five embryos from each stage and stained with toluidine blue.

From areas in which, from the semithin sections, the process of capillarisation of the CNS *anlage* was observed to have begun, thin sections were then cut with a Reichert or an LKB ultramicrotome for ultrastructural investigations. Finally these sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 109 electron microscope.

RESULTS

Light microscopy

In serial sections of paraffin-embedded mouse embryos, capillaries could be seen in the neuroepithelium for the first time on Day 9 plus 5 hours. At this point the embryos had reached the developmental stage, designated Stage 14 (Theiler, 1972). Two developmental markers of this stage are the closure of the anterior neuropore and the closure of the connection of the otic vesicles to the surface of the embryo. The areas of the CNS where the capillaries were initially formed were the prosencephalon, the rhombencephalon and the developing cervical spinal cord (Fig. 1). In the prosencephalon, capillaries occurred only in the dorsal part of the parieto-temporal wall. In the rhombencephalon, they were observed in the ventral part of the lateral wall. Whereas in the prosencephalon only a very small region was capillarised, capillaries were visible throughout the entire rhombencephalon right up to the region of the otic vesicles. From the longitudinal sections, it could be seen that capillarisation of the rhombencephelon and the spinal cord took place in such a way that it led to a segmentation of this part of the CNS (Fig. 2).

In the capillarised part of the developing cervical spinal cord, most of the capillaries entered the neural tube from the ventro-lateral side (Figs. 3, 5). It was interesting to note that, in the Stage 14 embryos investigated, the appearance of capillaries in the CNS *anlage* was not restricted to any one part of the neuroepithelium mentioned above. Where any vessels could be found, they were seen in the prosencephalon and the rhombencephalon, as well as in the developing cervical spinal cord.

In contrast to the results obtained on paraffin-embedded embryos, capillaries could already be observed in 1 μm sections of Epon-embedded embryos, at Stage 13 of development. At this stage, shortly after the turning of the embryo, the anterior neuropore was still open and the otic vesicles still had an open connection to the embryonic surface. In the serial sections of all four embryos at this stage, capillaries

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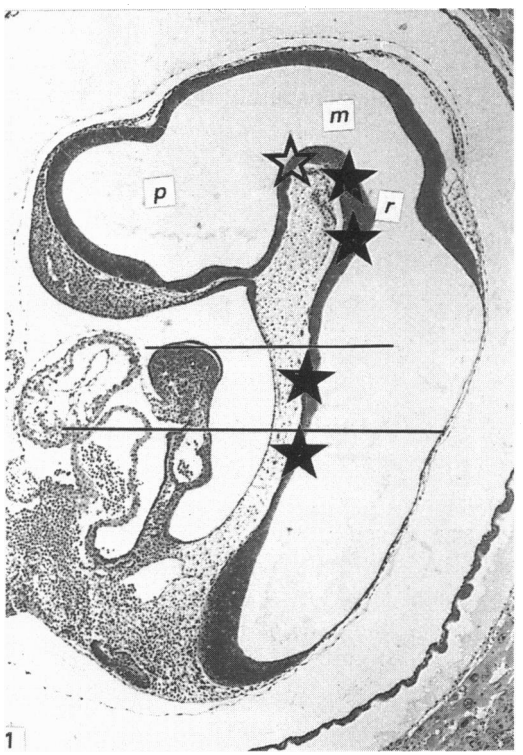
Fig. 1. Longitudinal section of a 9 days plus 5 hours mouse embryo (Theiler, Stage 14), *p*, prosencephalon; *m*, mesencephalon; *r*, rhombencephalon; black and open stars, areas of the CNS *anlage* in which capillaries could be localised at this developmental stage; open star, area of the prosencephalon in which, at Stage 13, first capillaries were seen in 1 μm sections (see Fig. 6*a*, *b*); transverse lines, planes of sections of Figs. 3 and 4. × 30.

Fig. 2. Longitudinal section through the rhombencephalon of a 9 days plus 5 hours mouse embryo; segmental capillarisation (arrows) from the ventrolateral side. *v*, lumen of the ventricle; *n*, neuroepithelial cell layer, × 250.

Fig. 3. Transverse section through the upper developing spinal cord of a 9 days plus 5 hours mouse embryo, arrows, capillaries mainly located in the ventro-lateral area; *v*, lumen of the ventricle; *n*, neuroepithelial cell layer, × 200.

Fig. 4. Transverse section through a 9 days plus 5 hours mouse embryo (Theiler, Stage 14) at the level of the heart *anlage* (*h*); square shows area of developing spinal cord from which Fig. 5 was taken. × 20.

Fig. 5. Higher magnification of area marked in Fig. 4. Sprouting of capillaries from the ventro-lateral side (arrows). *v*, lumen of the ventricle; *n*, neuroepithelial cell layer.



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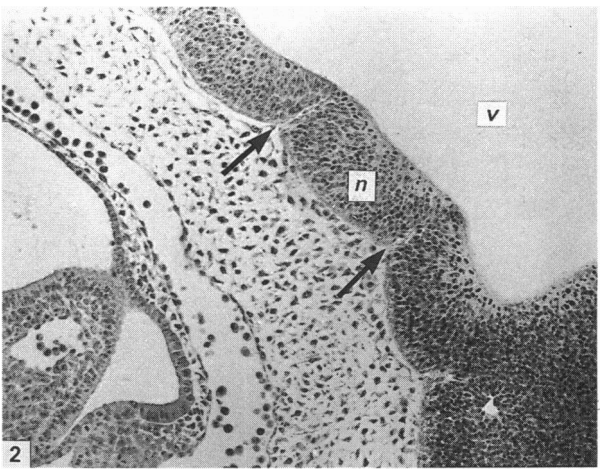

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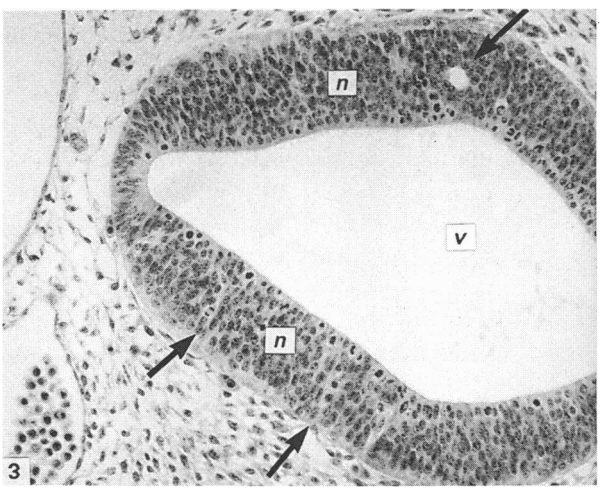

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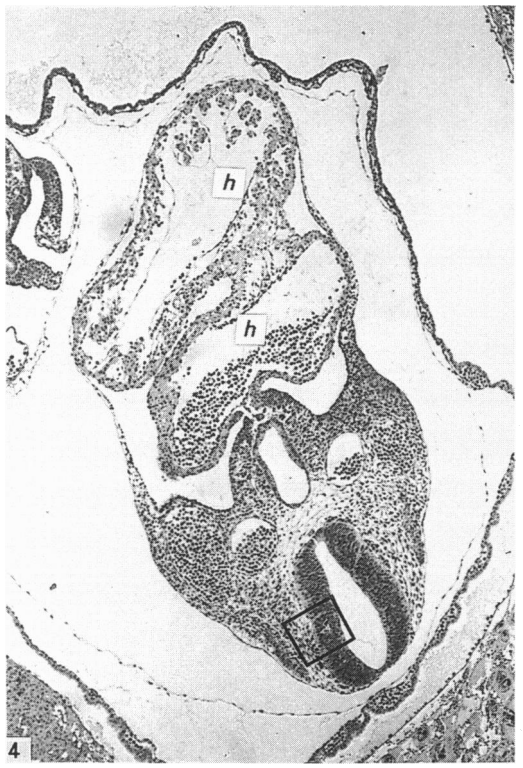

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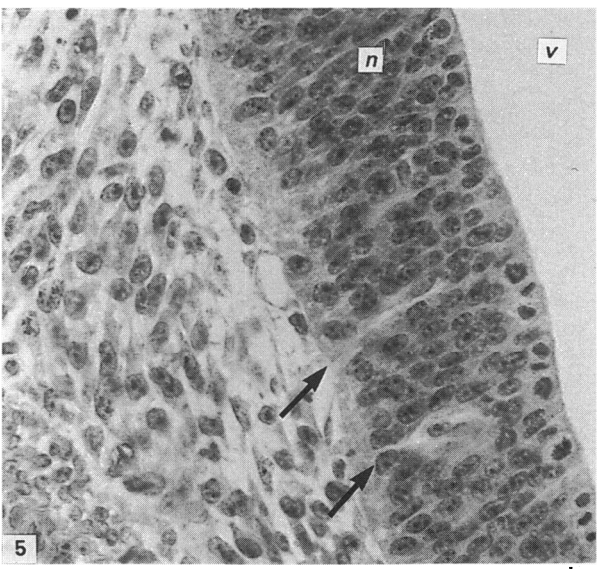

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were not seen in the developing spinal cord. In two of the four embryos, vessels could be observed in a very small lateral region of the dorsal prosencephalon and the ventral rhombencephalon. Moreover, in two of the embryos investigated, only one single capillary was seen on either side of the dorsal part of the parieto-temporal wall of the prosencephalon, indicating that this area is the part of the neuroepithelium in which capillarisation of the CNS *anlage* begins (Fig. 6).

Electron microscopy

At Stage 13 of development, when the first capillaries could be seen in the prosencephalon, the neuroepithelial cell layer was surrounded by a tannic acid-positive lamina densa layer of the basal lamina. The leptomeningeal capillaries, from which further capillaries sprout into the neuroepithelium, were not surrounded by a basement membrane at this stage.

At the ultrastructural level, the first indication that the process of capillarisation was beginning in the neuroepithelial cell layer, was an attachment of leptomeningeal capillaries to the neuroepithelium (Fig. 7). In those areas where leptomeningeal capillaries were attached to the CNS *anlage*, tannic acid-positive structures were seen between the endothelial cells of the capillaries and the neuroepithelial cell layer (Figs. 7, 8) and some fibres occurred in these areas between the fibrillar tannic acid-positive structures (Fig. 8). In the cytoplasm of the attached endothelial cells a very pronounced Golgi system was visible. Other organelles which occurred in these cells were ribosomes in polysome formation and some short fragments of a rough ER, but only a few mitochondria. Furthermore, some microtubules were visible in the cytoplasmic processes of the endothelial cells as well as in the cytoplasm near the nucleus. Microfilaments were only seen in a few endothelial cells on the abluminal side of the cytoplasm orientated towards the neuroepithelium.

A further ultrastructural marker indicating the process of capillarisation of the neuroepithelium was disturbance of the basement membrane of the CNS *anlage*. In those areas where the tannic acid-positive structures were located between the neuroepithelium and the attached endothelial cells, this disturbance of the basement membrane began with a thinning of the lamina densa, followed by rupture and disintegration of this layer of the membrane (Fig. 8). Shortly thereafter, processes of the endothelial cells of the attached leptomeningeal capillaries began to sprout into the neuroepithelium marking the start of capillarisation.

DISCUSSION

The aim of the present investigation was to define the beginning of the process of vascularisation of the CNS *anlage* of the mouse embryo. For such studies, one suitable method would be the injection of the embryo with India ink (Strong, 1964; Camosso,

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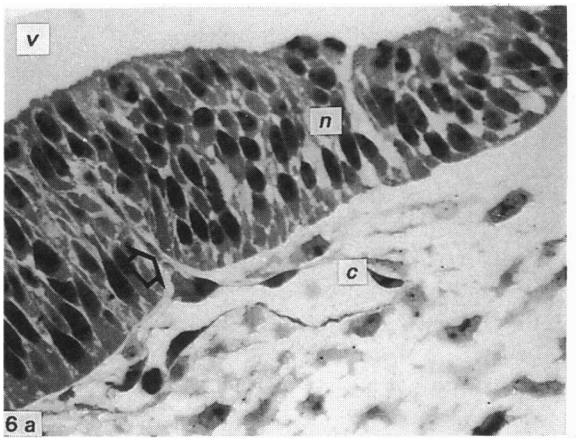
Fig. 6 (*a–b*). 1 μm cross-sections of right and left parts of the parieto-temporal wall of the prosencephalon of a 9 day plus 0 hours mouse embryo (Theiler, Stage 13). *v*, lumen of the ventricle; *c*, leptomeningeal capillaries from which the very first processes of endothelial cells (open arrows) penetrate the neuroepithelial cell layer (*n*). × 240.

Fig. 7. Leptomeningeal capillary attached to the neuroepithelial cell layer (*n*) of the prosencephalon of a 9 days old mouse embryo. Particularly noticeable are the tannic acid-positive structures (arrows) between the capillary and the neuroepithelial cell layer. *e*, endothelial cells of the capillary. × 5400.

Fig. 8. Higher magnification of the interstitial space between an endothelial cell of a leptomeningeal capillary and neuroepithelial cells (*n*) shortly before penetration of endothelial processes into the neuroepithelium. *L*, lumen of capillary; *G*, Golgi regions; stars, extracellular tannic acid-positive material; arrows, beginning of disintegration of the basement membrane. × 2300.



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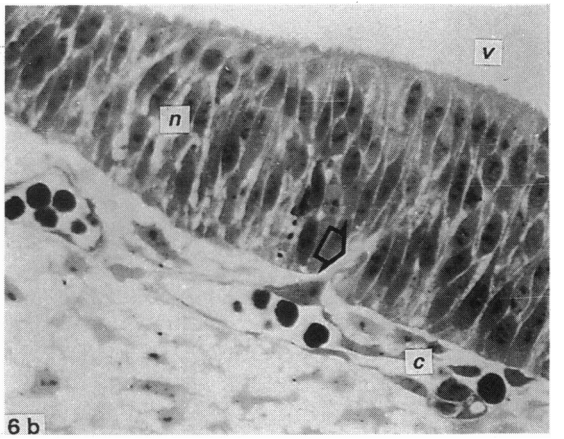

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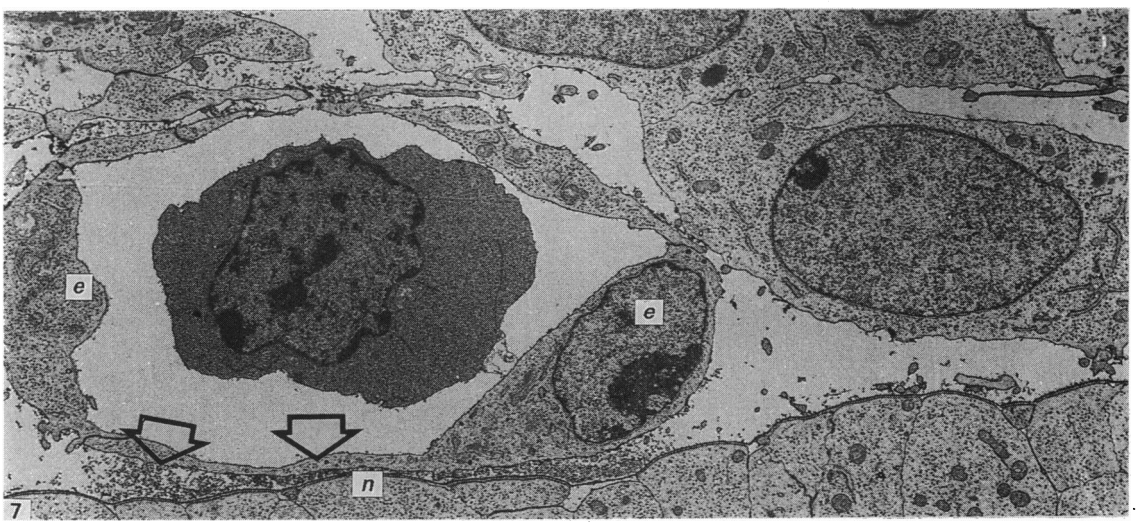

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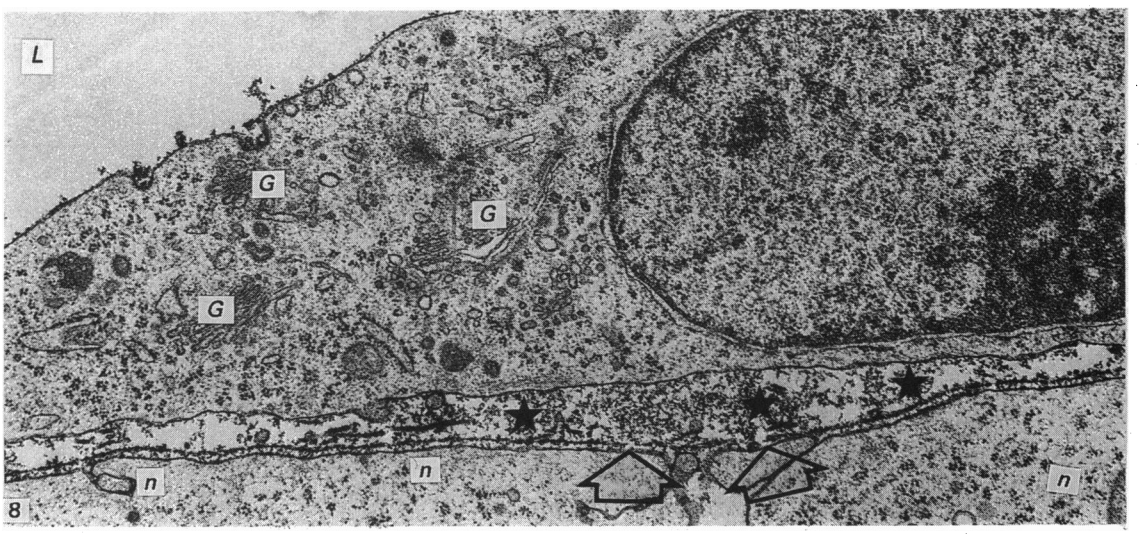

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Roncali & Ambrosi, 1976; Bär, 1980). However, we felt that this technique was not suitable for the determination of the earliest stage of capillarisation of the neuroepithelium. It takes some time before a growing capillary has developed to such an extent that blood containing the injected ink is able to pass through the lumen of the capillary. Therefore we investigated the appearance of the first capillaries in serial sections of embryos embedded in paraffin, so that the investigation was independent of the functional stage of the developing capillaries. For the screening of a number of embryos, paraffin embedding is still a suitable method because it allows cutting of serial sections in a relatively short time. A disadvantage of embedding in paraffin lies in the fact that serial sectioning of tissue using sections of less than 5 μm is difficult. Structures to be identified in the paraffin sections, in our case processes of endothelial cells, must therefore be larger than 5 μm.

Preliminary results obtained on such sections showed that capillaries were already visible in 9 days old, Stage 14 embryos. At this developmental stage, capillaries were seen in the prosencephalon, the rhombencephalon and the developing cervical spinal cord. This would suggest that vascularisation of the CNS *anlage* begins at the same time in all three parts of the CNS.

However, prompted by the findings of other authors who have demonstrated the advantage of semithin sections for an accurate determination of the beginning of the process of vascularisation (Sturrock, 1981; Simon-Marin *et al.* 1983), we supplemented our findings by using serial sections of resin-embedded embryos. With the help of our preliminary results obtained with paraffin sections, we could then limit the time-consuming cutting of 1 μm resin sections to embryos of the early Day 9 of development.

Unexpectedly, the initial cytoplasmic processes of capillaries could be clearly recognised in the neuroepithelial cell layers of mouse embryo, already at Stage 13 starting in the parieto-temporal wall of the prosencephalon. One should bear in mind that at this very early stage of embryonic development, on the early part of Day 9, the embryo has only just turned and the neural tube has just closed, the anterior neuropore still remaining open.

The acceptance of the appearance of cytoplasmic processes of endothelial cells sprouting into the neuroepithelial cell layer as an indication of the onset of vascularisation of the CNS *anlage* implies acceptance of the theory that the intraneural vessels derive from the leptomenigeal capillaries and not from angioblastic stem cells within the neural tissue. However, the results of experiments on chick-quail chimeras provide evidence that intracerebral vessels originate from the sprouting of extra-cerebral vessels into the neural tissue (Stewart & Wiley, 1981).

This study was not only aimed at determining the morphology of when and where the first capillaries occur in the embryonic CNS of the mouse but was also undertaken to investigate those changes at the ultrastructural level which are indicative of the beginning of vascularisation. An interesting result was the appearance of tannic acid-positive stained extracellular structures between the neuroepithelial cell layer and the adjacent endothelial cells of the leptomeningeal capillaries which had started to sprout into the neuroepithelium. Some of the known effects of tannic acid fixation are the precipitation of glycoproteins and glycosaminoglycans and the formation of complexes between carbohydrates and osmium (Sannes, Katsuyama & Spicer, 1978). It has been shown that tannic acid fixation stains numerous extracellular matrix components such as hyaluronic acid (Sanders, 1979), proteoglycans (Singley & Solursh, 1980), fibronectin (Singer, 1979) and the basement membrane components laminin and collagen Type IV (Herken & Barrach, 1985). Any of these extracellular

matrix components could be involved in the initial spreading of leptomeningeal capillaries into the neural tissue at the beginning of the process of vascularisation of the embryonic CNS *anlage*. Further immunohistochemical investigations must be undertaken to identify the biochemical nature of these components.

SUMMARY

The developmental stage of the mouse embryo at which capillaries first occurred and their localisation in the neuroepithelium were investigated on serial sections of 9 and 10 days old embryos embedded in paraffin and Epon. In addition, areas of the neuroepithelium in which capillaries had been observed at the light microscopical level were investigated by electron microscopy carried out on embryos fixed with glutaraldehyde supplemented with tannic acid.

In 5–7 μm serial sections of paraffin-embedded embryos, capillaries were initially seen in the CNS *anlage* at Theiler's Stage 14 (1972). At this stage, capillaries also occurred in the prosencephalon, the rhombencephalon and in the developing spinal cord.

In 1 μm serial sections of resin-embedded embryos, capillaries could be identified in the neuroepithelium one stage earlier, i.e. at Stage 13. These very early capillaries were seen in the dorsal part of the lateral wall of the prosencephalon which later forms the diencephalon.

At the ultrastructural level, those areas of the neuroepithelium in which leptomeningeal capillaries first started to spread into the neuroepithelium were characterised by the disintegration of the basement membrane of the neuroepithelium and the appearance of tannic acid-positive extracellular structures between the neuroepithelium and the adjacent leptomeningeal capillaries.

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