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Microvascular architecture in the central nervous system of *Ambystoma mexicanum* (Caudata, Ambystomatidae). A light, and transmission and scanning electron microscopy study

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ABSTRACT. A combination of light, and scanning and transmission electron microscopy of vascular corrosion casts was utilized to study the structure and spatial organization of intraparenchymal microvessels in the central nervous system of the urodele *Ambystoma mexicanum* (Shaw, 1789). The angioarchitectural pattern consists of both single and paired vessels, but the single ones are prevalent. The meningeal vessels give origin to both single and paired elements. The paired vessels are bent and twisted and extend into the neural wall to various depths. Their two limbs are apposed throughout their length and are connected by a U-shaped terminal loop. The cross-sectioned single and paired vessels have variable diameters. The hairpin-shaped vascular pairs do not branch into secondary loops and no anastomotic intraparenchymal connections are observed with single or paired neighbouring structures. All intraparenchymal vessels are structurally interpreted as capillaries. The paired vessels of *Ambystoma mexicanum* resemble those found in a few vertebrates whose brain vascular pattern is represented only by paired structures.

KEY WORDS : Amphibia; central nervous system; vasculature; capillary; corrosion casting.

INTRODUCTION

Each organ has a peculiar angioarchitectural pattern, which is related to the functions of its different tissues and of the whole organ. The structural and spatial aspects of the central nervous system (CNS) vessels have been studied in various vertebrates (Bubis & Luse, 1964; Bundgaard, 1982; Lazzari & Franceschini, 2000), but conventional techniques based on light microscopy have failed to visualize both the general three-dimensional organization and the fine structure of tissue vasculature. Investigations of the spatial disposition of microvessels were difficult until a method based on scanning electron microscopy (SEM) observation of methyl methacrylate plastic casts was devised (Murakami, 1975). The use of a new low-viscosity plastic resin in combination with the high resolving power and the great depth of field of the SEM stimulated studies on the microangioarchitecture in many tissues and organs (see Christofferson & Nilsson, 1990; Lametschwandtner et al., 1990).

To date there have been several studies on the vascular features of anuran brains, mostly using the corrosion casting technique (see Lametschwandtner et al., 1980; Albrecht et al., 1980; Lametschwandtner, 1982 for references), whereas SEM research and ultrastructural studies on the brain vasculature of caudates mainly concern *Triturus carnifex* (Laurenti, 1768) (Ciani & Franceschini, 1984; Franceschini et al., 1988; Lazzari et al., 1991). The only information about the brain vasculature of *Ambystoma mexicanum* (Shaw, 1789) is reported by Roofe (1935) and more recently by Ciani et al. (1989) in a cytochemical investigation. These preliminary studies on *Ambystoma mexicanum* report the presence of a vascular model of the CNS that differs from the two main patterns of CNS vascularization found in vertebrates. These works examine neither the ultrastructure nor the overall three-dimensional organization of these vessels. It appears important to investigate these aspects as they could provide information on the evolution of CNS blood supply.

Therefore, this study was undertaken to elucidate the angioarchitectural pattern and the ultrastructural features of CNS microvessels in *Ambystoma mexicanum* by a combination of light, and transmission and scanning electron microscopy techniques. The results will be useful for comparative analyses of brain vascularization patterns, not only in the amphibians, but also in other vertebrates.

MATERIALS AND METHODS

Animals

Twenty-four metamorphosed *Ambystoma mexicanum* of both sexes (70-85 g body weight, 19-23 cm total length) were obtained from neotenic animal stock raised in our Department. Metamorphosis was induced by Eutirox (Bracco, Milano, Italy) : 2-3 pills were administered over a period of a week inside bits of meat. The urodeles were maintained in terra-aquaria at room temperature in a natural light-dark cycle and fed *ad libitum* with bits of

meat. All procedures were in accordance with the guidelines of the European Communities Council Directive (86/609/CEE), the current Italian legislation for the use and care of animals, and conform to the guidelines of the U.S. National Institute of Health. This study was also approved by the Ethic-Scientific Committee of the University of Bologna. The amphibians were sacrificed by immersion in 0.1% 3-aminobenzoic acid ethyl ester (Sigma Chemical Co., St. Louis, Missouri), and the blood was removed from the vascular system by intracardiac perfusion with 50 ml of Holtfreter's physiological solution containing heparin (3 IU/ml). They were perfusion-fixed with 150 ml of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). All solutions were kept at 4˚C and propelled by a Gilson Minipulse 3 peristaltic pump operating at a constant flow of 5 ml/min.

Light microscopy

The brains and spinal cords of eight urodeles were removed and immersion-fixed in the same fixative used for perfusion for an additional 4 hours at 4˚C. After washing overnight in 0.1 M phosphate buffer (pH 7.4) at 4˚C, they were dehydrated with ethanol and embedded in Paraplast Plus (Sherwood Medical, Athy, Ireland; melting point 55-57˚C). Coronal 10 µm thick sections were mounted on poly-L-lysine (Sigma Chemical Co., St. Louis, Missouri) coated slides and dried. The sections were deparaffinized, hydrated, stained with haematoxylin-eosin solution and then coverslipped with Permount (Fisher Scientific Co., Pittsburgh, Pennsylvania).

Transmission electron microscopy

After washing overnight in 0.1 M phosphate buffer (pH 7.4) at 4˚C, the brains and spinal cords of eight amphibians were coronally-sectioned into 100 µm thick tissue slices using a Vibratome 1000 (Lancer). The slices were then postfixed in 0.1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 hour at 4˚C. After a buffer rinse, tissue slices were dehydrated in acetone and embedded in Durcupan ACM (Fluka Chemical Co., Buchs, Switzerland). Semithin sections, 0.5-1 µm thick, were stained with toluidine blue. Thin sections were collected on formvar-coated copper grids, stained with uranyl acetate and lead citrate solutions and examined in a CM 100 Philips electron microscope operating at 80 kV.

Scanning electron microscopy

Eight urodeles, perfusion-fixed as above, were processed for vascular corrosion casting. After their fixation, 60 ml of freshly prepared low-viscosity methyl methacrylate (Gannon, 1981) were injected manually at a constant rate through a cannula inserted in the conus arteriosus at room temperature. After 30 min the injected specimens were placed in a water bath at 60˚C overnight to accelerate and complete the resin polymerization. Afterwards, the heads were immersed in 20% KOH at 60˚C for 24 hours for soft tissue ablation. The solution of KOH was replaced every 6 hours and the endocast washed with a fine stream of water to remove eroded tissue fragments still adhering to the plastic surface. Bone decalcification was then carried out with 2.5% HCl. Finally the casts were treated with 5% aqueous solution of trichloroacetic acid to remove any product of saponification still adhering, and thoroughly rinsed several times in distilled water. The clean replicas were frozen in distilled water at -20˚C and the ice blocks were freeze-dried to prevent vessel collapse (Lametschwandtner et al., 1990). The casts were mounted on aluminium stubs by means of conductive bridges with silver conductive glue, gold-coated in a Bio-Rad SC 502 SEM Coating System and examined in a JEOL JSM-5200 scanning electron microscope at an accelerating voltage of 15 kV.

RESULTS

Light microscopy

All the regions of the *Ambystoma mexicanum* CNS had a parenchymal vascularization with both single and paired vessels (the single ones being prevalent), irregularly arranged throughout the neural wall (Fig. 1A, B). The vascularization arose from the meningeal vessels, from which originated both single and paired elements (Fig. 1C, D). Although the initial track of the microvascular pairs penetrated into the nervous parenchyma almost perpendicularly to the brain surface, they seldom had a rectilinear course in the nervous tissue and the majority of them were twisted and curved (Fig. 1D), which made them appear transversely or obliquely cut in the CNS sections. In each vascular pair the two adjacent limbs were connected by a narrow U-shaped terminal loop (Fig. 1E). The hairpin-shaped vascular pairs extended into the CNS wall to various depths. Some penetrated from the meningeal layer into the superficial nervous tissue for a short distance, others reached deeper layers penetrating into the gray matter and sometimes contacted the ependymal layer.

In tissue sections the two limbs of a pair did not link with the surrounding single or paired vessels. Moreover hairpin-shaped vascular loops did not appear to branch.

Transmission electron microscopy

Both single and paired vessels were present in the nervous tissue (Fig. 2A, B). The paired structures consisted of two adjoining vessels leaning against each other and separated by a thin wall (Fig. 2B). In the various vascular pairs the two adjacent vessels presented the same structure. Both paired and single vessels had similar ultrastructural characteristics. The vascular wall was unfenestrated, and the endothelial cells were thin except in the nuclear region (Fig. 2A, B, C). The nucleus appeared flattened and elongated with chromatin masses placed adjacent to the nuclear envelope. In the paired vessels the nucleus could be located in any part of the 8-shaped vascular outline, and could even enter into the intervascular wall (Fig. 2D). The endothelial lumenal surface had numerous short, finger-like protrusions, which extended in the peripheral zone of the vascular lumen (Fig. 2D, E, F). In the endothelial cells, organelles were mainly located in the region next to the nucleus, where the endothelium thickness increased (Fig. 2C). A few vesicular profiles were free within the endothelial cytoplasm, others were confluent with the endothelial surface membranes appear-

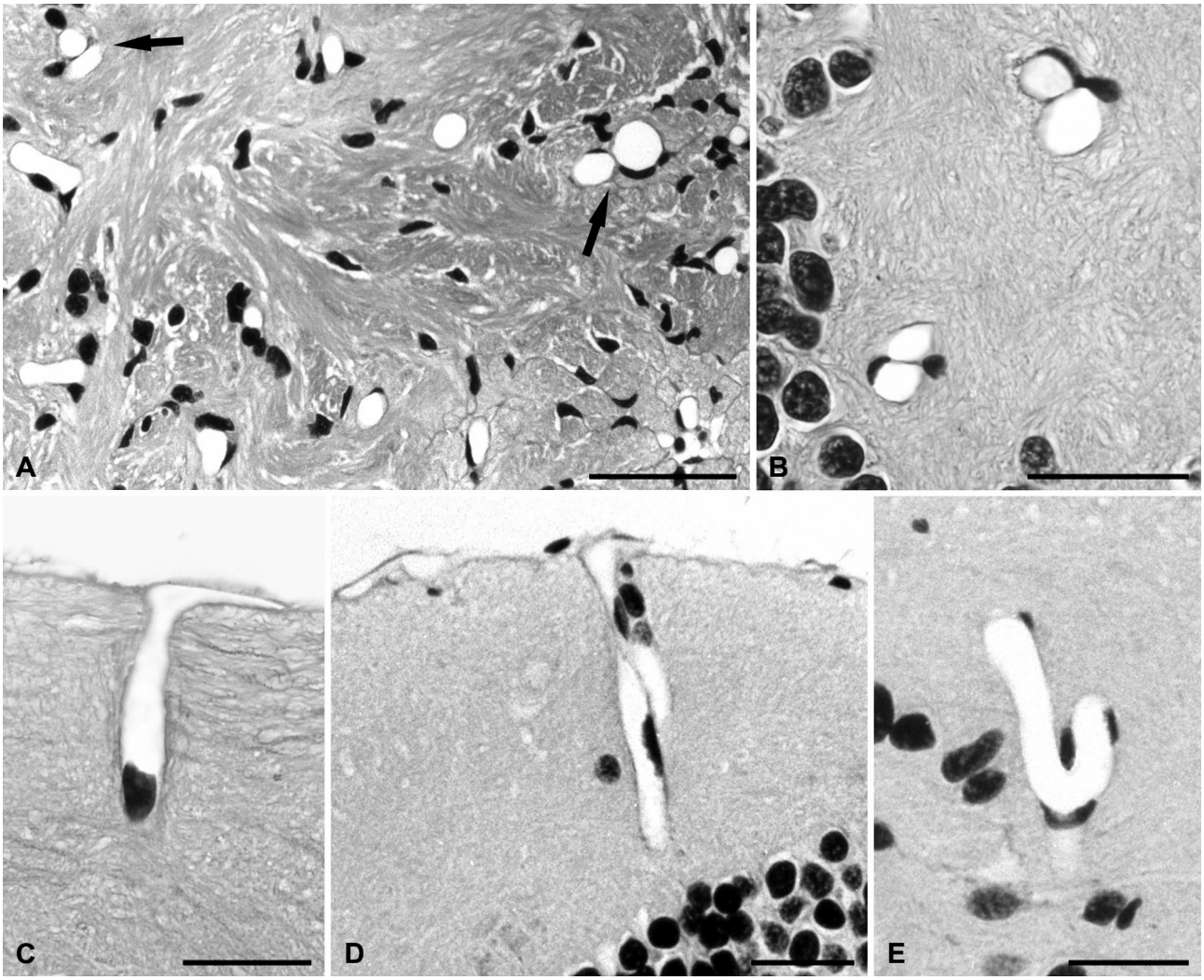

Fig. 1. – Micrographs of *Ambystoma* CNS sections showing parenchymal microvessels. **A** : A few paired vessels (arrows) appear among the single vascular elements in the olfactory bulb. Scale bar, 100 µm. **B** : Two transversely sectioned vascular pairs in the deepest region of the telencephalic white matter. Scale bar, 50 µm. **C** : A single microvessel originates from the meningeal vasculature in the optic tectum. Scale bar, 50 µm. **D** : A pair of tightly apposed, twisted and radially-oriented microvessels originates from the meningeal vasculature in the diencephalon. Scale bar, 50 µm. **E** : A terminal endloop connects the adjacent microvessels of a vascular pair in the medulla oblongata. Scale bar, 50 µm.

ing as pits (Fig. 2E, F). The overlapping endothelial cell edges were connected by tight junctions (Fig. 2C). Neither single nor paired vessels had a smooth muscle cell investment, but sometimes both were associated with pericytes whose processes extended into the endothelial basal membrane. These cells had a nucleus and cytoplasm similar to those found in endothelial cells, but did not constitute a continuous covering. In particular, in the paired vessels, pericytes sometimes appeared to be wedged between the adjacent vascular elements (Fig. 2D, F) with thin processes entering into the thinner part of the intervascular wall (Fig. 2E).

In both single and paired vessels the neuropilar side of the endothelial basal membrane was lined almost continuously by a layer of perivascular glial endfeet that appeared flattened on the basal membrane and were rich in mitochondria (Fig. 2C, D, F). In the paired vessels these perivascular glial cell processes were never found entering into the wall separating adjoining elements; therefore this wall was generally composed of the thin endothelium of each adjacent vessel separated by a single basal membrane. A slight increase in thickness could be due not only to the presence of pericytic cell processes but also to somata in the basal membrane or to endothelial nuclei in one or both endothelial sides.

The same ultrastructural characteristics were found in tissues from both sexes.

Scanning electron microscopy

The features of the vascular pattern were uniform in the different areas of the *Ambystoma mexicanum* CNS in both sexes as revealed by whole vascular casts as well as dissected region moulds. In the vascular casts at the pial surface the meningeal vessels appeared as single elements with a rather winding course (Fig. 3A). They generally forked repeatedly with different angles into two smaller branches, which finally gave rise to the vascular network below (Fig. 3B). The intraparenchymal vessels had a similar branching pattern (Fig. 3C) and sometimes revealed a

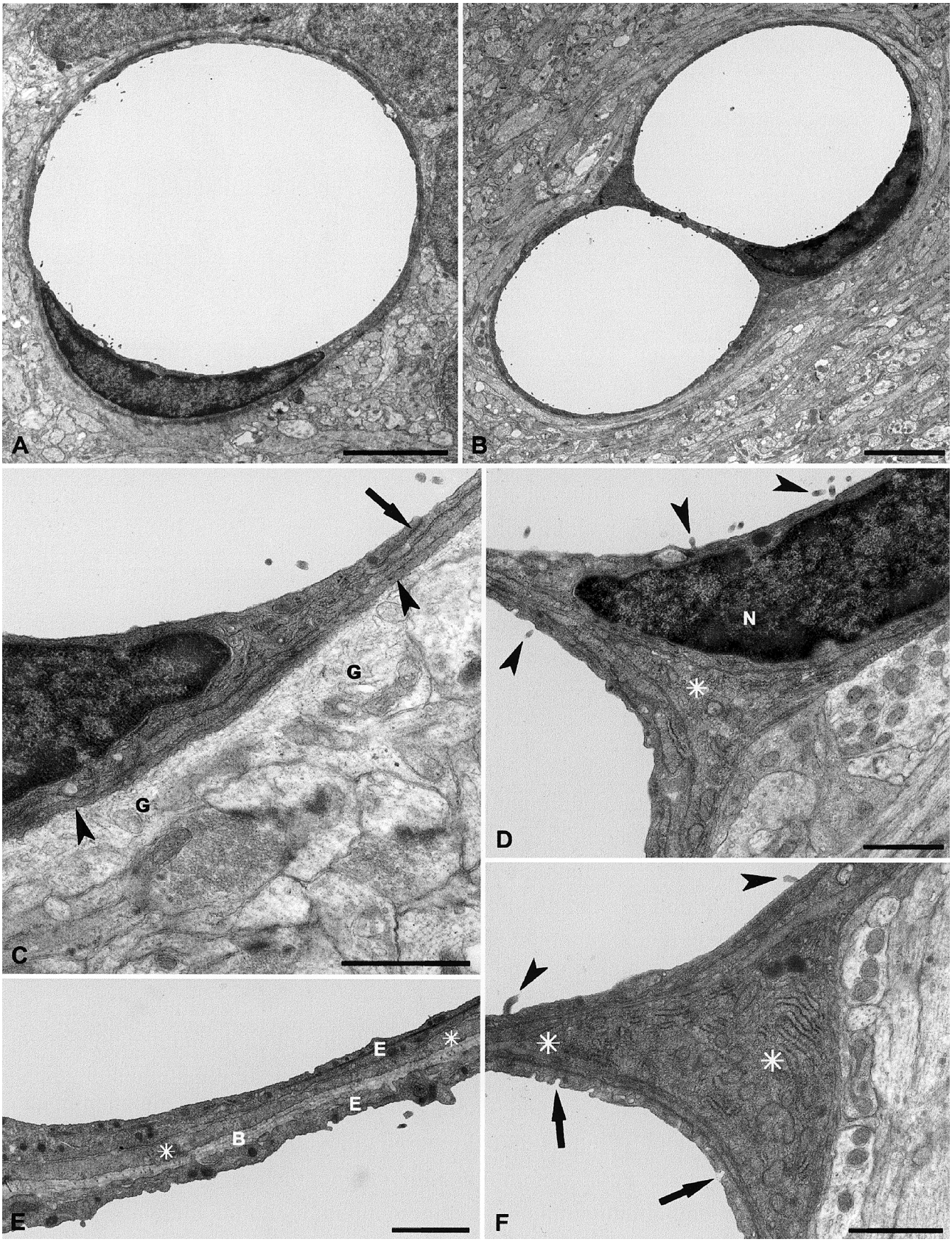

Fig. 2. – Transmission electron micrographs of *Ambystoma* CNS microvessels. **A** : A transversely-sectioned single vessel in the telencephalon showing an elongated and flattened endothelial cell nucleus. Scale bar, 5 µm. **B** : Transverse section of a telencephalic vascular pair with the two closely applied limbs showing a slightly different size. An endothelial cell nucleus is clearly observed in one vascular limb. Scale bar, 5 µm. **C** : Glial endfeet (G) line the endothelial basal membrane (arrowheads) of a mesencephalic single vessel. A tight junction (arrow) links overlapping endothelial cell edges. Scale bar, 1 µm. **D** : An endothelial nucleus (N) penetrates into the thickest tract of the intervascular wall, which includes a pericytic cell process (asterisk). Some finger-like protrusions (arrowheads) arise from the luminal endothelial surface. Scale bar, 1 µm. **E** : A pericytic process (asterisks) enters into the intervascular wall. Scale bar, 1 µm. B, basal membrane; E, endothelial layers. **F** : A pericytic cytoplasmatic mass (asterisks) extends laterally between the two adjacent vessels and penetrates into the intervascular wall. Pits (arrows) and finger like protrusions (arrowheads) appear on the endothelial luminal surface. Scale bar, 1 µm.

winding route with U-shaped loops connected in series (Fig. 3D).

In the vasculature of all CNS regions numerous paired vessels also appeared penetrating from the meningeal surface. They were irregularly spaced in relation to both themselves and the single elements, and were variously curved and inclined with respect to the meningeal surface. In each vascular pair the two elements were apposed all along their routes with a narrow U-shaped loop that connected the two limbs, giving rise to a hairpin-like structure (Fig. 3E, F). Vessels were variously bent and twisted. In the vascular corrosion casts, SEM observations did not show direct anastomoses either between the two closely applied limbs or with adjacent vascular loops and single vessels. The hairpin-shaped vascular pairs were not ramified.

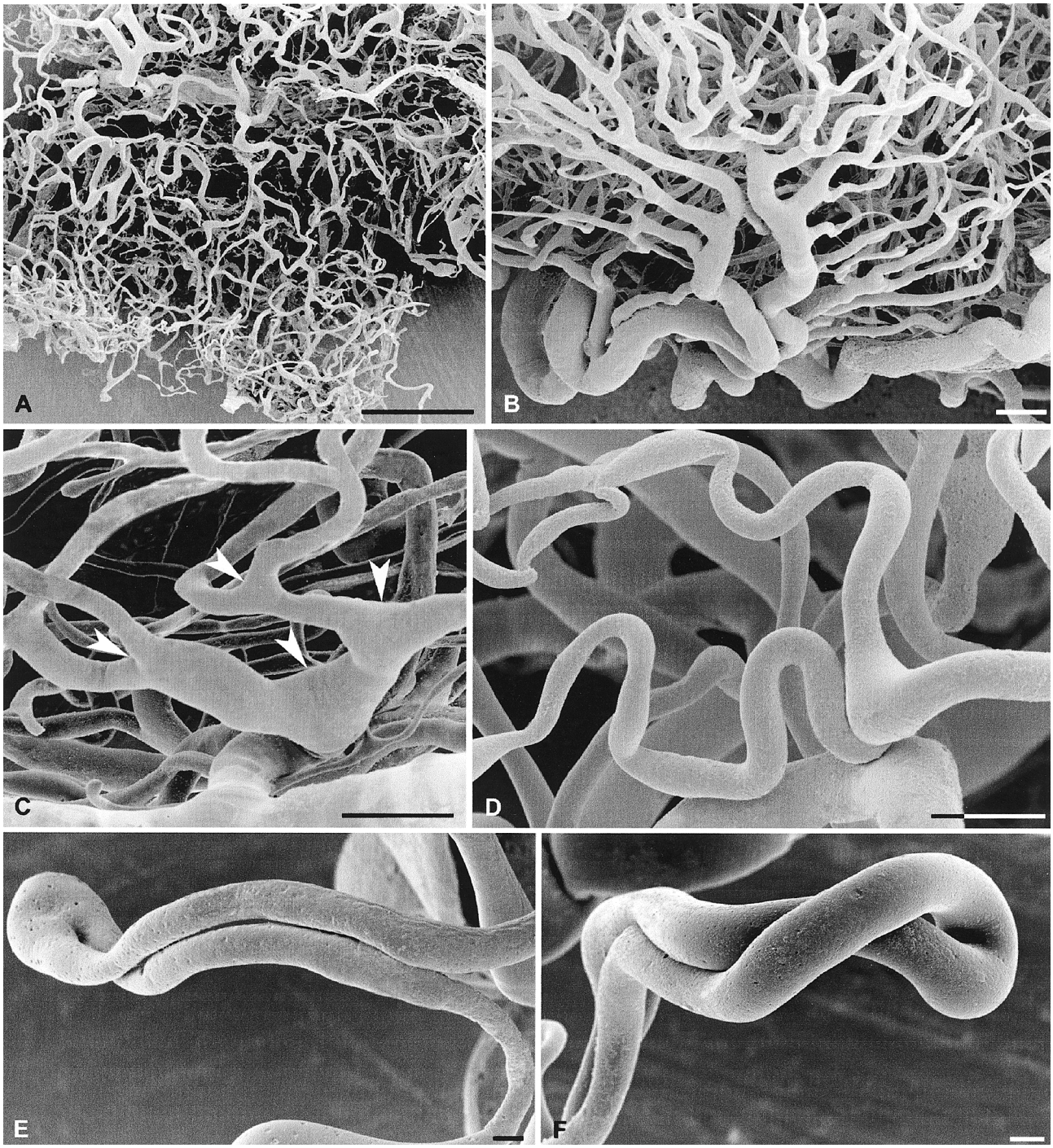

Fig. 3. – Scanning electron micrographs of microvascular corrosion casts of *Ambystoma* CNS. **A** : Dorsal view of the posterior telencephalon. The meningeal vessels are single elements in their winding course. Scale bar, 500 µm. **B** : The single meningeal vessels divide repeatedly in the lateral mesencephalic region. Scale bar, 100 µm. **C** : Parenchymal single vessels fork repeatedly (arrowheads) in the spinal cord. Scale bar, 100 µm. **D** : Wide U-shaped loops in single telencephalic vessels. Scale bar, 50 µm. **E** : A hairpin-like vascular structure of the medulla oblongata formed by closely apposed paired microvessels connected by a narrow U-shaped endloop. Scale bar, 10 µm. **F** : A mesencephalic vascular pair is bent and twisted. Scale bar, 10 µm.

DISCUSSION

Studies on the vertebrate CNS have revealed two main microvascular patterns. The most diffuse condition is represented by single vessels anastomozed in a complex three-dimensional network. It is found in eutherian mammals, turtles, fishes and anurans (Honma et al., 1987; Naka et al., 1987; Snyder et al., 1990a; Kleiter & Lametschwandtner, 1995). The second pattern is formed by paired microvessels dipping into the nervous tissue from the meningeal layer and terminally connected by a narrow U-shaped endloop. They constitute hairpin-like vascular structures that show no anastomotic connections with the adjacent vascular pairs. This condition is present in cyclostomes (Bundgaard, 1982) and widely represented in amphibians belonging both to the Gymnophiona (Craigie, 1940, 1941a) and the Urodela (Lazzari et al., 1991). As for reptiles, hairpin-like loops have been described in Sauria in studies with conventional light microscopy methods (Craigie, 1941b) and more recently by a TEM-SEM combination in Scincidae (Snyder et al., 1990b) and Lacertidae (Lazzari & Franceschini, 2000). The paired vessel pattern has also been pointed out in marsupials (Wislocki & Campbell, 1937; Bubis & Luse, 1964; Snyder et al., 1989).

A few other vertebrate species belonging to different classes have both single vessel meshwork and paired vessel loops in their CNS : the dipnoan *Epiceratodus forsteri* (Krefft, 1870) (Craigie, 1943), the crocodile *Alligator mississippiensis* (Daudin, 1802) (Craigie, 1941c) and *Ambystoma mexicanum* (Craigie, 1938, 1939; Bodenheimer & Brightman, 1968; Ciani et al., 1989). These studies are not recent, are mostly based on light microscopy techniques, and only the latest ones utilize TEM observations. As for *Ambystoma mexicanum*, these studies did not examine in detail the ultrastructural aspect of paired vessels compared to single ones, nor their distribution and the overall microvascular pattern of the CNS using SEM. The present study reports for the first time the ultrastructure and spatial organization of the vessels belonging to a model of CNS vascularization that is structurally intermediate between single vessel meshwork and paired vessel loops.

In *Ambystoma mexicanum* we found both single and paired vessels, but the first clearly prevailed. Their structural features identify them as capillaries. In particular, vascular pairs and single vessels show the same structure in transverse sections independently of their size, and no smooth muscle investment could be detected in TEM observations. This is in accordance with the condition of cyclostomes (Bundgaard, 1982) and urodeles (Bodenheimer & Brightman, 1968; Ciani & Franceschini, 1984), which have only paired vessels in their CNS. Marsupials and scincids, however, have hairpin-like capillary loops, which come off arterioles and venules that are themselves paired into the CNS (Snyder et al., 1989, 1990b). This different condition could be related to different CNS masses and, especially in marsupials, to the larger thickness and more complex neuronal organization of the brain.

In the CNS vascularization of *Ambystoma mexicanum*, age- and individual-based variations were not observed, particularly there was no difference between the vascular pattern in the CNS of a mature neotenic specimen and a metamorphosed one (Ciani, unpublished data).

Microscopic and submicroscopic observations showed that in *Ambystoma mexicanum,* hairpin-shaped loops do not branch into secondary ones. The simplest branching pattern, which is a secondary loop formed only by the contribution of one limb of a primary vascular pair, was found in Gymnophiona (Craigie, 1940), in *Triturus carnifex* (Lazzari et al., 1991), as well as in the lizard *Podarcis sicula* (Rafinesque, 1810) (Lazzari & Franceschini, 2000). This pattern is characterized by a very short secondary loop generally close to the terminal endloop. We hypothesize that this pattern relates to increased needs for blood supply in the tissue layer that is located in the deepest part of the neural wall next to the ependyma and brain ventricles. The other branching pattern, found in *Podarcis sicula* (Lazzari & Franceschini, 2000) and formed by the contribution of both capillary limbs of a primary pair, reminds us of marsupial hairpin capillary loops that nevertheless originate from closely paired intraparenchymal arterioles and venules (Snyder et al., 1989). Both branching patterns in the paired microvessel model meet major trophic demands of the neural tissue, and their absence in *Ambystoma mexicanum* could be related to trophic need satisfaction by the three-dimensional single vessel network.

According to Snyder et al. (1989) it is possible that paired capillaries play an important role in oxygen exchange in the nervous tissue by modifying diffusive gas conductance. These authors suppose that paired capillaries may operate as counter-current exchangers for carbon dioxide, the concentration of which in the blood becomes higher than normal levels. This could cause a large Bohr shift and release oxygen from the paired capillary blood. This event would raise the oxygen tension in the nervous tissue surrounding the vascular pair and increase the distance from the vascular pair to which the tissue could be adequately provided with oxygen. Nevertheless specific studies on gas exchange at the level of the paired capillaries of the CNS are still required to verify these assumptions.

In *Ambystoma mexicanum*, whose condition is intermediate between the capillary loop pattern and single vessel network organization, the closely related functional and phylogenetic significances are still unclear. According to Craigie (1938, 1940) the capillary loop condition represents an ancestral state with little adaptive significance, from which the single vessel network has developed. On the contrary, Wislocki & Campbell (1937) suggested a specific functional adaptation based on a counter current exchange process rather than a simple ancestral organization. Nevertheless Craigie (1938) considered it improbable that an adaptive evolution to paired brain microvessels would have occurred in various vertebrates differently located phylogenetically if it were not a return to a more primitive condition.

Snyder et al. (1989) compared the condition of a marsupial that has only paired vessels in its CNS, the northern native cat, *Dasyurus hallucatus* (Gould, 1842), with the condition of an eutherian mammal, the rat, which has only a single vessel network. These authors suggest that satisfying increased requests for gas exchange during

evolutionary development of the CNS may have required abandoning paired vessel pattern in favour of anastomotic meshwork. In this regard, *Ambystoma*, showing both vascular patterns and particularly with the anastomotic meshwork prevailing on the paired vessel loops, would present a CNS vascularization more evolved than the condition of Gymnophiona and most Urodela, which show only paired vessel loops. Moreover the condition of *Ambystoma* would be less evolved than the anuran one, which shows only single vessels in the CNS.

The present study shows that the structure and organization of hairpin-shaped paired vessels of *Ambystoma mexicanum* are the same as in vertebrates that have the pair pattern exclusively : cyclostomes (Bundgaard, 1982), gymnophiones (Craigie, 1940, 1941a), urodeles (Bodenheimer & Brightman, 1968 ; Ciani & Franceschini, 1984), lizards (Snyder et al., 1990b ; Lazzari & Franceschini, 2000) and marsupials (Bubis & Luse, 1964 ; Snyder et al., 1989). Moreover in newts (Ciani & Franceschini, 1984 ; Lazzari et al., 1991), lizards (Lazzari & Franceschini, 2000), and marsupial northern native cat (Snyder et al., 1989), as well as *Ambystoma mexicanum* (present study), the two paired limbs appear closer than in opossum CNS in which cells of unknown origin and function separate the two elements of a loop (Bubis & Luse, 1964). The most important feature of *Ambystoma mexicanum* paired vessels and of the vertebrates that possess only paired vessels in their CNS (cyclostomes, gymnophiones, newts, lizards and marsupials) is that the perivascular glial processes never enter into the thin intercapillary wall that separates the two adjacent limbs (Bubis & Luse, 1964 ; Bodenheimer & Brightman, 1968 ; Bundgaard, 1982 ; Ciani & Franceschini, 1984). The fact that both vascular loops and single vessels are found in all regions of the CNS would indicate that the functional needs that promoted the transition from paired vessel pattern to anastomotic meshwork operated in the whole CNS.

Many studies on various vertebrate species showed that brain capillaries are involved in the blood-brain barrier (see Brightman, 1992 ; Brightman & Tao-Cheng, 1993 ; Vorbrodt, 1993). Its main structural bases are the lack of fenestration, general absence of free transendothelial passages, and, particularly, the presence of tight junctions sealing adjacent endothelial cells and preventing the diffusion of substances such as electron microscopic tracers. Pits of the endothelial plasmalemma and vesicular profiles in the cytoplasm may be involved in transcytosis through the endothelial cells (Pardridge, 1993). Even though direct functional studies on vascular permeability to microscopical tracers and histochemical localization of enzyme activities have not yet been carried out in the brain capillaries of *Ambystoma mexicanum*, the ultrastructural features here reported are in accordance not only with the condition of *Triturus carnifex* (Ciani & Franceschini, 1984) and lizards (Shivers, 1979 ; Lazzari & Franceschini, 2000), which show a paired vessel pattern, but also with the findings in eutherian mammals (Brightman, 1992), which have a three-dimensional brain capillary network.

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