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Microscopical and Ultrastructural Investigations on the Development of the Blood-Brain Barrier in the Chick Embryo Optic Tectum

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Summary. The formation of a blood-brain barrier to horseradish peroxidase was microscopically and ultrastructurally investigated in the tectum opticum of the chick during development of the intraneural blood vessel network from the 6th incubation day to hatching, and in adult specimens.

Extravasation of the circulating marker, apparently unimpeded during early stages of vasculogenesis, starts to diminish from the 14th incubation day (i.d.) and is prevented after the 18th i.d. The tracer seems to get out of the vessel lumina through the sites of reciprocal contact between adjacent endothelial cells, and the differentiation of tight junctions there hinders the passage of peroxidase particles. The formation of numerous endothelial vacuoles during early vasculogenesis and the setting of the blood-brain barrier are discussed in connection with the mechanisms of transendothelial transport, and respectively, the processes of moulding of the growing endothelia.

Key words: Chick embryo – Optic tectum – Blood-brain barrier – Horseradish peroxidase

Introduction

The morphological constituents of the blood-brain barrier have been described in detail both under normal and experimental conditions, while the role played by each of them individually (interendothelial junctions and basement lamina, phagocytic pericytes, perivascular astrocytic endfeet) in the functioning adult barrier is still debated (Van Deurs 1980).

With the aim of investigating this problem, the developmental features and the temporal sequence in the appearance of the blood-brain barrier components are analyzed, and a comparison is drawn between the modifications of ultrastructure and those of permeability of the developing blood vessels in the tectum opticum of the chick embryo. The vascular permeability is studied using an exogenous peroxidase (horseradish peroxidase) as a tracer according to the Reese and Karnovsky (1967) method. This has already been applied in many studies on the mature (Van Deurs 1980) as well as on the developing (Peterson 1970; Wakai and Hirokawa 1978) blood-brain barrier.

Development of the tectum vessels and ultrastructural differentiation of their walls have been analyzed already in previous researches under normal and experimental conditions (Roncali et al. 1985a, b, c). Preliminary reports on our findings have been presented at the 5th International Meeting of the International Society for Developmental Neuroscience (Roncali et al. 1984a) and at the 3th World Congress for Microcirculation (Roncali et al. 1984b).

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Material and Methods

One hundred and twenty fertilized chicken eggs were incubated from the start of their embryogenesis in an incubator under conditions of constant humidity at a temperature of 37.5° C. Three to six embryos of 6, 10, 12, 14, 17, 18 and 21 days of incubation and four 30-day-old chickens were utilized, killed by decapitation 10–20 min after an intracardial injection of horseradish peroxidase (HRP) (0.3 mg Sigma type II HRP per gram body weight in 0.1–0.3 ml saline solution). The optic tecta, removed from the head of each specimen, were sectioned according to coronal planes and fragments therefrom were fixed by immersion in a sodium cacodylate-buffered mixture of 2% glutaradehyde and 2% paraformaldehyde, then washed for 12 h in the same buffer. Thick sections (50 μm), cut from each tectum fragment with a vibratome, were incubated for 30 min at room temperature in a 0.05% solution of 3′,3′-diaminobenzidine in 0.05 M Tris-HCl buffer (pH 7.6), containing 0.01% H_2O_2. After incubation, some of the thick slices were dehydrated, mounted on slides and examined with a SM lux Leitz microscope, others were post-fixed in 1% osmium tetroxide, and embedded in Epon 812. A number of slices after post-fixation were stained in toto for 1 h in 2% uranyl acetate in sodium maleate buffer. Semithin

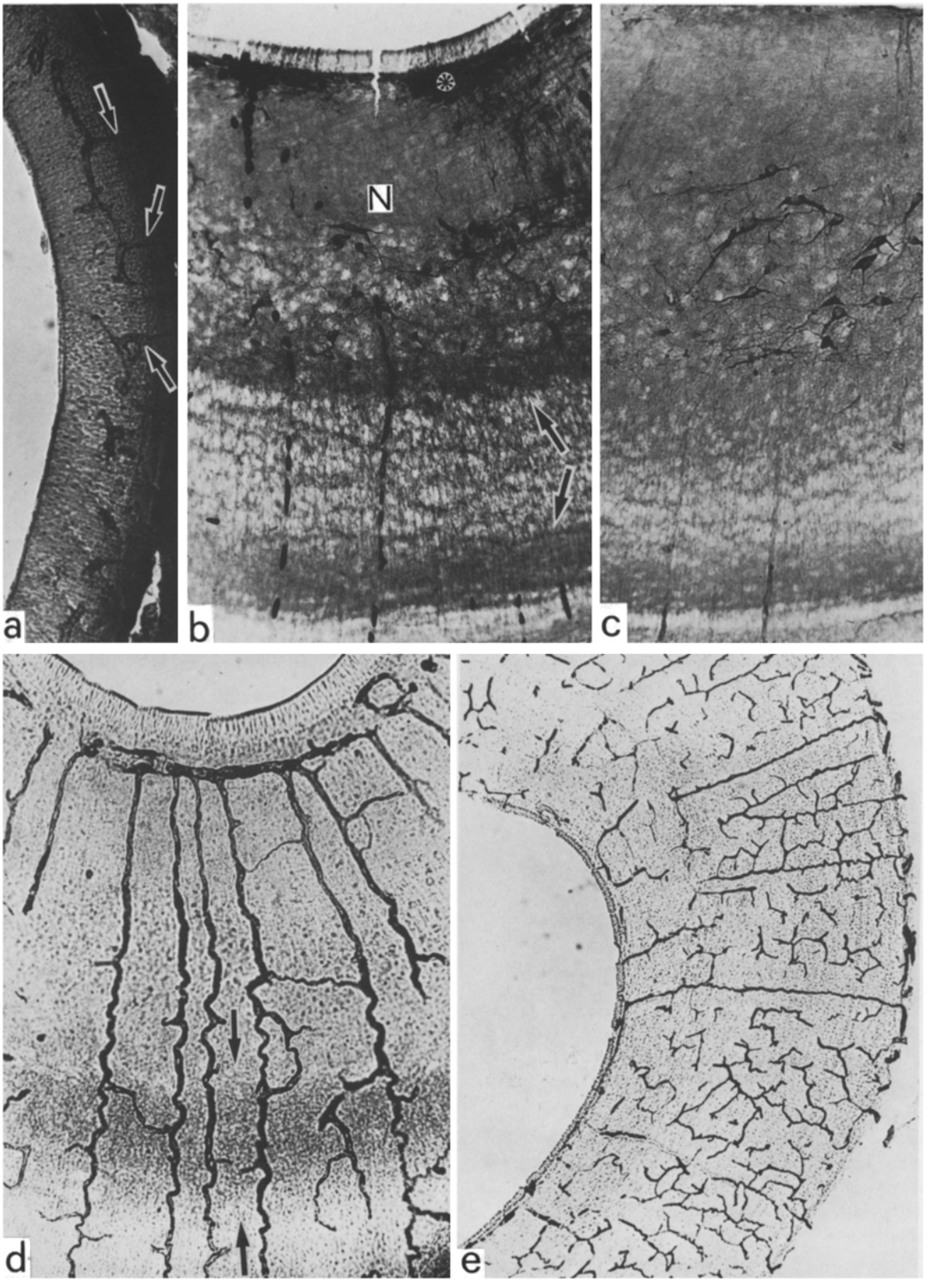

Fig. 1

and ultrathin sections were cut with an LKB V ultramicrotome; the semithin sections were stained with toluidine blue, the ultrathin, stained with lead citrate, were examined under a 9A Zeiss electron microscope.

Control optic tecta of embryos (two for each developmental stage), and 30-days chickens (three specimens) injected with saline solution, were subjected to the same procedures for microscope and electron microscope study.

Peroxidase activity is apparent as a reaction product, which is brown in the thick sections and intensely electron opaque in the ultrathin slices. Some features of the developing vascular patterns in the chick optic tectum could also be seen because of the peroxidase-like activity of the hemoglobin of erythrocytes.

Results

Microscopical Observations

In the thick sections of optic tecta fixed at the 6th, 10th and 12th incubation day (i.d.), the marker reaction product displays a prevalent extravascular localization. At the 6th i.d., when the tectum is built of a thick neural epithelium enveloped by a thin mantle layer and its intrinsic vascular pattern consists of radial vessels connecting an external plexus to a newforming deep one, the tracer is uniformly spread throughout the neural wall (Fig. 1a). At the 10th and 12th i.d., collateral branches of the radial vessels establish mutual interconnections so building up large intratectal meshes within the thick mantle layer, in which 10–12 laminae of neuroblasts and nerve processes alternate. The tracer is mostly found outside the vessels of the deep plexus and in regions of the tectal wall characterized by high cell density, so that the gray matter laminae, particularly the sixth and the eight layers, and strands of cell groups migrating through the mantle layer are clearly evident. The localization of the peroxidase in the neural substratum is extracellular, between neuroblast bodies and processes, as well as intracellular, large multipolar neurocytes of the fourth layer being stained by the reaction product of the tracer (Fig. 1b, c).

At the 14th i.d. the amount of HRP activity through the tectum opticum is less than in preceding days, since extravascular spreading of the marker is notably reduced. The cell and fiber layers of the tectum are not easily identifiable in the thick sections as they are unstained almost everywhere and only the fourth, sixth and eight gray laminae are slightly tracer-labeled (Fig. 1d). The confinement in the vessel lumina of the HRP seems to be independent of the location of the blood vessels which, at the 14th i.d., form a network throughout the mantle layer.

In 17-day specimens a small amount of tracer diffuses only from the subependymal deep plexus and, by the 18th i.d., peroxidase activity is exclusively confined within the vessels of the capillary intraneural meshwork. Neither neurocytes, gliocytes nor pericytes and pericapillary glial endfeet appear to be marked (Fig. 1e).

Electronmicroscopical Observations

In the specimens fixed at the 6th and 10th i.d. the vascular lumina are nearly deprived of HRP, whose reaction product coats the endothelium lining, albeit irregularly owing to plasmamembrane invaginations, microvillous expansions and marginal folds (Fig. 2a).

The intraendothelial localization of the HRP is considerable inside large vacuoles, coated by the marker, and small pinocytotic vesicles, filled up by it (Fig. 2b), sometimes merging the ones with the others. The vacuoles are rarely seen opening in the subendothelial spaces. In the regions of contact between adjacent endothelial cells the marker fills the intercellular clefts along their whole length from the luminal to the abluminal surfaces of the endothelium (Fig. 2 c, d). HRP is also present in the space between endothelium abluminal surface and pericytes, and inside large phagocytic vacuoles of their bodies and processes (Fig. 3). In the neural substratum the reaction product of the marker is apparent as tiny granules among nerve cell bodies and axons (Fig. 3) and, at the 10th i.d., inside large vacuoles of well-differentiated multipolar neurocytes of the tectum fourth layer.

At the 14th i.d. the endothelium of the blood vessels is covered by a thin layer of HRP reaction product contained within vacuoles and small vesicles. In the tectum regions, in which the microscopical observations do not reveal extravasation of HRP, interendothelial junctions, characterized by scattered punctuated contacts between the facing plasmamembranes, seem to check the transendothelial passage of

Fig. 1. Thick slices of optic tectum at the 6th (**a**), 10th (**b**), 12th (**c**), 14th (**d**) and 18th (**e**) incubation day (i.d.). **a** Intraneural vessels (*arrows*) radially directed, after bifurcating within germinal layer, anastomose one to another to form a deep network. Their lumina are nearly free from horseradish peroxidase (HRP) reaction product, whereas the latter is uniformly distributed throughout the nerve wall; ×80. **b** Peroxidase activity, very intense in the subependymal nervous substratum (*asterisk*), makes apparent large neurons of the cellular layer fourth (*N*) and the densely packed sixth and eighth cellular laminae (*arrows*). The course of the vessel lumina is revealed by the intense peroxidase activity of the red blood cells; ×180. **c** Neurons of the fourth layer; ×180. **d** The HRP reaction product fills the whole vascular network of the tectum; the slight HRP activity of the cellular components of the sixth layer (*arrows*) indicates a reduced extravasation of the marker; ×180. **e** Throughout the tectum the tracer is confined to the vessel lumina; ×65

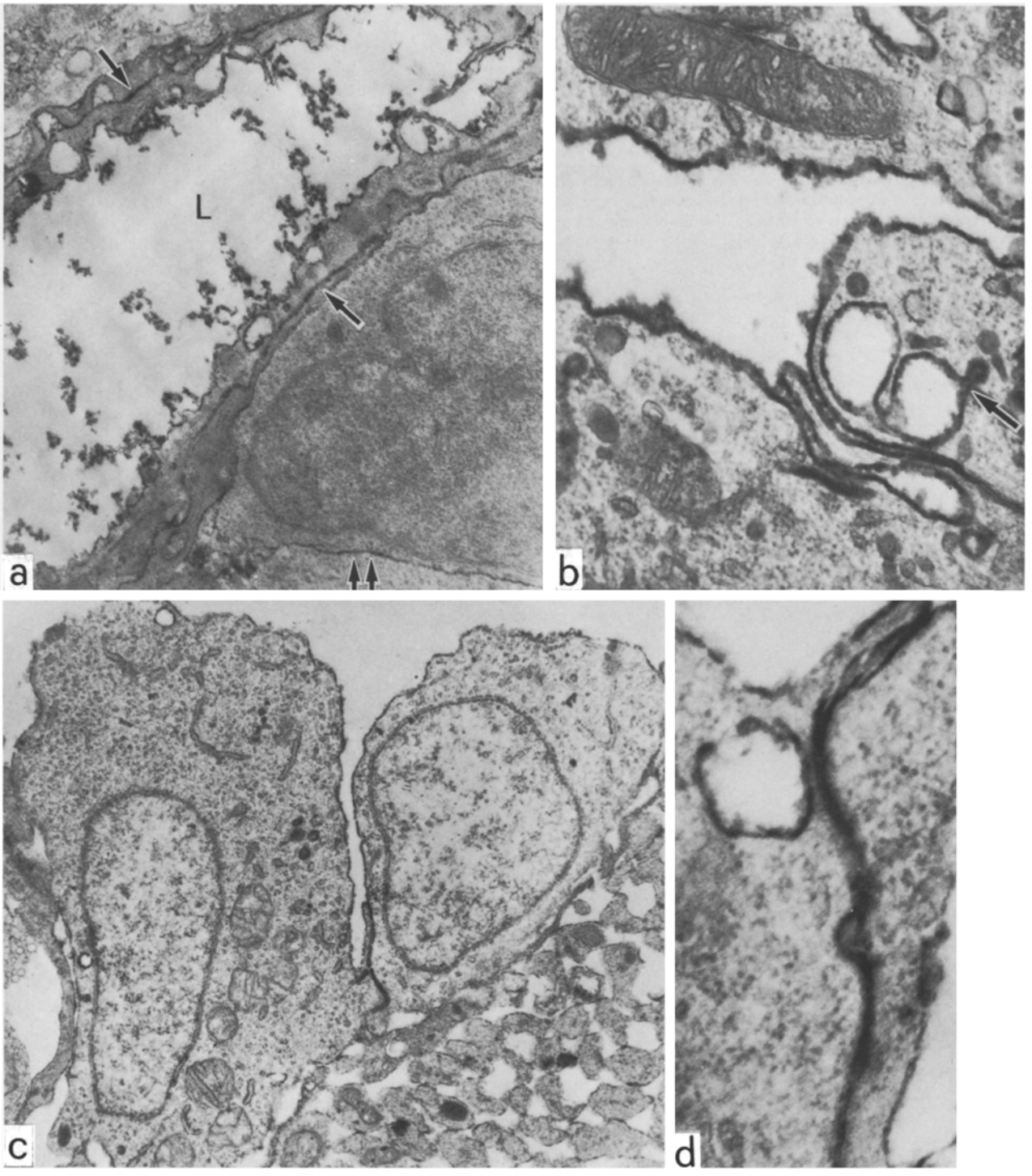

Fig. 2. **a** 10th i.d. HRP activity is detectable in the vessel lumen (*L*), close by the endothelial lining, in the subendothelial spaces (*arrows*) and between the bodies of adjacent neurocytes (*double arrow*); ×11,400. **b** 10th i.d. A vesicle and two tracer-labeled vacuoles merging into each other (*arrow*); ×36,000. **c** 6th i.d. Two endothelial cells interconnected by a junction marked by the HRP reaction product; ×11,400. **d** 6th i.d. Interendothelial cleft filled by the tracer; ×5,000

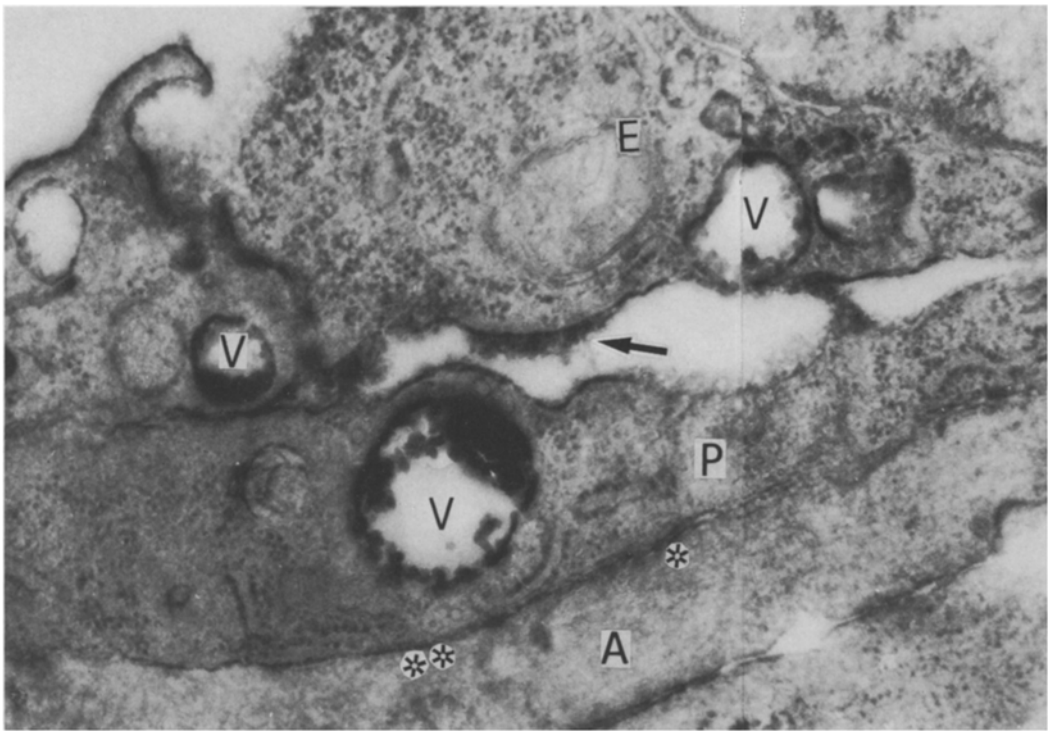

Fig. 3. 10th i.d. HRP inside endothelial (*E*), pericyte (*P*) and vacuoles (*V*), in the subendothelial space (*arrow*) and between a pericyte and an adjacent axon (*A, asterisks*); ×38,000

the marker and the new-formed endothelial basement lamina is never tracer-labeled. On the contrary, in the tectum parts characterized, in thick sections, by extravascular diffusion of peroxidase, the intercellular clefts of the endothelium appear invaded by the marker product, and the latter is also identifiable among the nervous and glial components (Fig. 4a, b, c).

At the 18th and 21th i.d. the vessel lamina are filled by the HRP. The endothelial surface is smooth and vesicles, varying in size and full of tracer, are more frequently located closer to the luminal than to the abluminal aspect of the endothelial cells (Fig. 5a, b). Such vesicles often appear merging with, but never discharging their content into, the subendothelial clefts. The clefts between the marginal expansions of neighboring endothelial cells, overlapping for long distances and welded by rows of fusion spots of the two plasmalemmas, are devoid of tracer (Fig. 5c). No significant differences are found as regards the HRP localization between 18-day embryos and adult chickens (Fig. 5d).

Discussion

The data of the present study related to the development of the vascular pattern in the chick embryo optic lobes are in agreement with those of previous research carried out on the same CNS region by means of intravascular India ink injections (Roncali et al. 1985a).

The morphological analysis of the formation of the blood-brain barrier to horseradish peroxidase shows that at the 6th, 10th and 12th i.d. the tectum microvessels are completely permeable to this tracer, which after intracardial injection leaves the vascular bed almost entirely. Throughout the mentioned developmental period the vessel endothelial lining is particularly irregular displaying endoluminal and basal expansions and invaginations, and vacuoles of various size. The contact regions between adjacent endothelial cells appear invaded by the tracer reaction product. At the 14th i.d. the endothelium luminal and abluminal irregularities are still evident, but scattered spots of tight contact connecting the plasmamembranes of contiguous endothelial cells seem to prevent the marker extravasation. Shortly before hatching, the endothelial surfaces are smooth and the HRP is exclusively intraluminal and intraendothelial. The overlapping thin peripheral expansions of the endothelial cells are coupled by junctions now built of rows of punctuated contacts between the external leaflets of the facing plasmalemmas. Even if these data do not seem completely satisfactory and suitable for ascertaining whether the marker extravasation up to the 14th i.d. occurs by a transendothelial mechanism

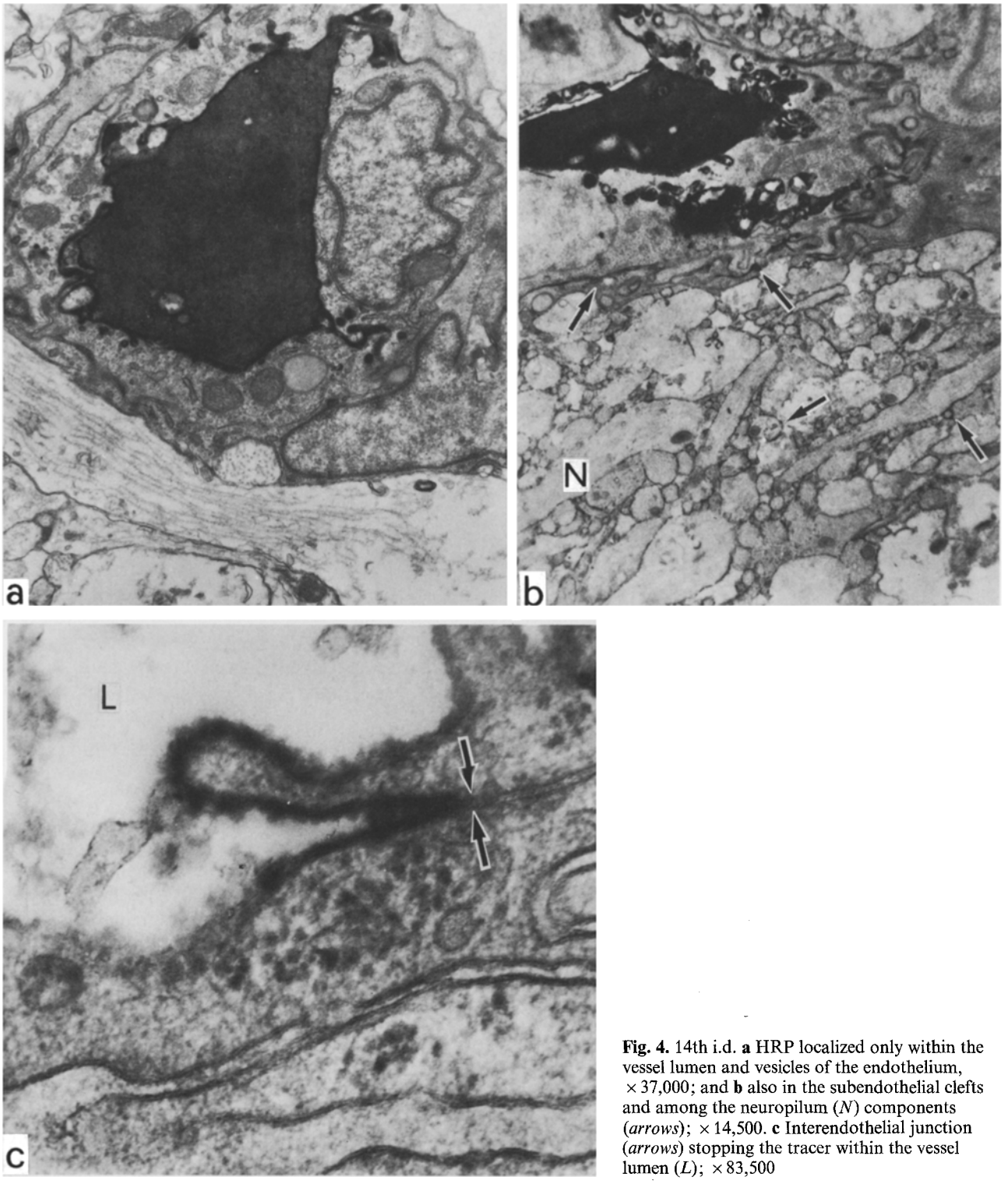

Fig. 4. 14th i.d. **a** HRP localized only within the vessel lumen and vesicles of the endothelium, ×37,000; and **b** also in the subendothelial clefts and among the neuropilum (*N*) components (*arrows*); ×14,500. **c** Interendothelial junction (*arrows*) stopping the tracer within the vessel lumen (*L*); ×83,500

(endo-exocytosis) or, through the clefts between neighboring endothelial cells or, perhaps, by both mechanisms, some suggestions can be drawn from the results. A relatively scarce number of vesicles in the cerebral adult endothelia (Reese and Karnovsky 1967; Brightman et al. 1983; Cervos-Navarro et al. 1983; Roncali et al. 1985b, c) might be one of the morphological features of the functioning blood-brain barrier, whereas the richness in vacuoles, vesicles and luminal and abluminal expansions and invaginations dis-

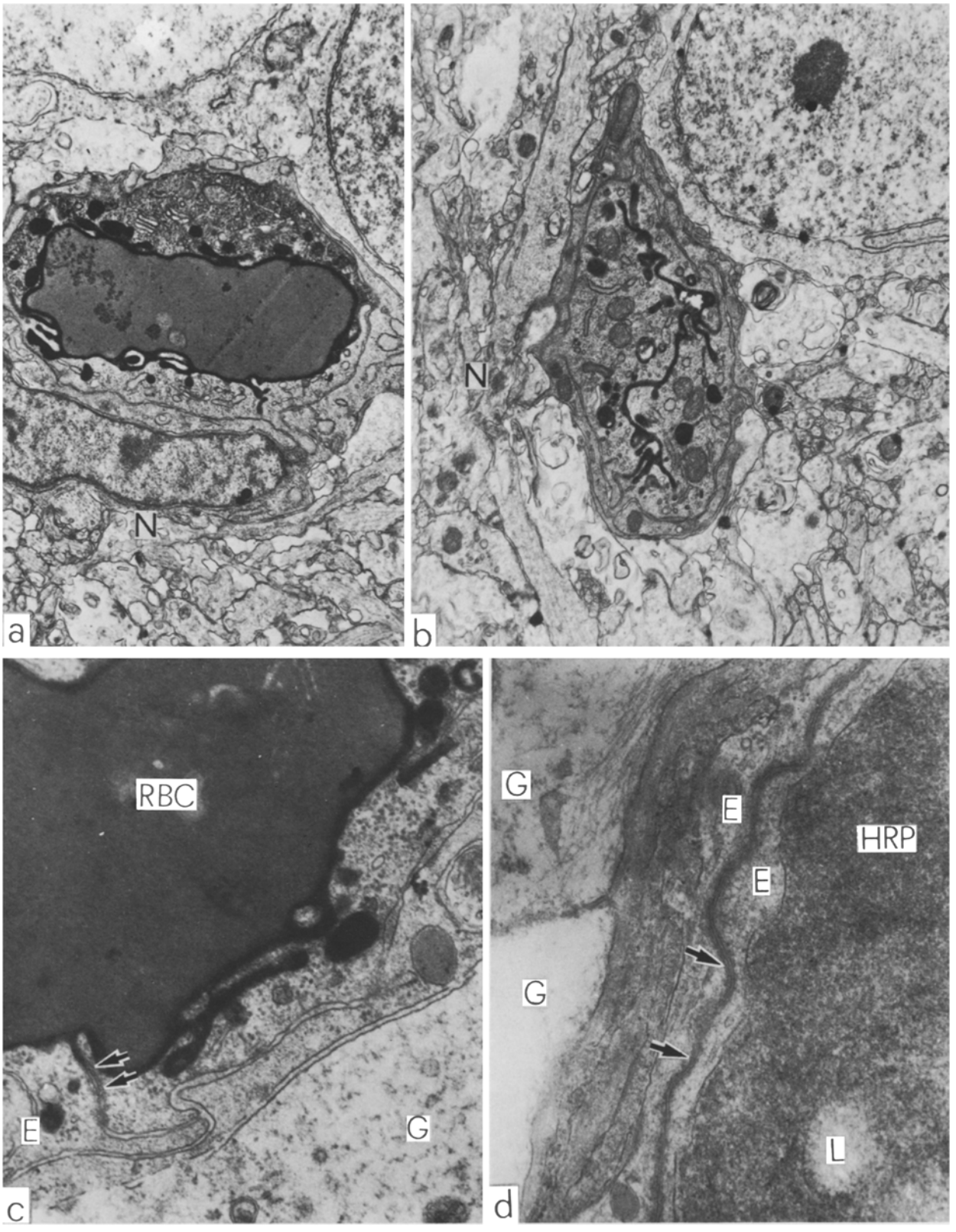

Fig. 5. a and **b** 18th i.d. The neuropilum (*N*) surrounding the blood vessels is free from tracer. HRP fills the vesicles of the endothelium; × 11,650 **a** and **d**. Tight junctions (*arrows*) between apposed expansions of endothelial cells, at the 18th i.d. **c**, and in an adult chicken **d**. *E*, endothelial cell; *L*, vessel lumen; *RBC*, red blood cell; *HRP*, horseradish peroxidase; *G*, glial process. **c**, **d** × 37,000

played by endothelial cells of immature cerebral, and non-cerebral, microvessels (Bertossi and Roncali 1981; Bertossi et al. 1983; Roncali et al. 1985b) could be indication of a high endo- exocytotic activity allowing transendothelial transport. As regards the spreading of the HRP through the wall of the tectum microvessels during the first half of embryonic growth, our observations seem to indicate that it does not occur, or not exclusively, by means of endo- exocytosis mechanism. The emptying speed of the tectal vessels, which soon after HRP injection show only a thin layer of the marker close to the endothelial lining, as well as the constant presence of tracer in the interendothelial clefts lead to the conclusion that, during the early vasculogenesis of the tectum, the marker's passage into the perivascular spaces and the neuropilum occurs intercellularly. Endo- exocytosis mechanism might take part in the tracer extravasation, but they could be involved in a lesser degree and at a later time than the mechanism of diffusion through the intercellular clefts; actually, the intraendothelial vacuoles observed at the 6th and 10th i.d. are only coated, and never filled, by the HRP reaction product and they are rarely seen opening into the subendothelial clefts. The irregular course of the luminal and abluminal aspects of the endothelium during early vasculogenesis could also be referred to the plasticity and mobility of the growing endothelia of the new-forming vessels, on account of the fact that these features undergo reduction during differentiation of the vessel walls, while the rate of the vasculogenetic processes decreases (Bär and Wolff 1972; Bär 1980; Bertossi and Roncali 1981; Bertossi et al. 1983; Roncali et al. 1985b).

As it occurs in the cerebral capillaries of the adult and in some experimental and pathological situations (Giacomelli et al. 1970; Lorenzo et al. 1972; Brightman et al. 1973; Van Deurs 1976; Brightman 1977; Cervos-Navarro et al. 1983), the interendothelial junctions seem to be the stopping site of the marker, beginning from the 14th i.d., and thus the morphological expression of the building up of the blood-brain barrier during vasculogenesis of the tectum. In fact, not even traces of the marker are detectable along the intercellular clefts when tight junctions are identifiable (Staehelin 1974; Simionescu et al. 1975) as isolated punctuated contacts, from the 14th i.d., and then as rows of fusion spots between contiguous endothelial cells, from the 18th i.d.

These results on the temporal sequence in the blood-brain barrier formation suggest that differences would exist in its establishment during central nervous system organogenesis in the chick embryo, since in the cerebellum and spinal cord rudiments the barrier is already fully formed by the 15th i.d. (Wakai and Hirokawa 1978).

The pericytes, periendothelial cells of the cerebral microvasculature, apparently not involved in the functioning of the blood-brain barrier, surround the microvessels from the start of the tectum vaculogenesis (Roncali et al. 1985b) and phagocyte large amount of marker before the establishment here of a blood-brain barrier to HRP; so, they could play the role of a periendothelial barrier before an endothelial one is formed (Kristensson and Olsson 1973).

In conclusion, while the close relationship between formation of a blood-brain barrier and, respectively, of tight junctions during vessels development in the chick embryo tectum seems to be firmly established, it should also be pointed out that when the blood-brain barrier to horseradish peroxidase begins to be efficient an endothelial basement lamina has just become well evident and, moreover, astrocytic endfeet have commenced to surround the endothelium and pericyte layers (Roncali et al. 1985b).

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