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Absence of blood vessels in the brain parenchyma of hynobiid salamanders[1]

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Summary. The brains of hynobiid salamanders were studied with a light and an electron microscope. Blood vessels were found in the neural lobe, the olfactory bulb, and the meninx. In the bulk of the brain parenchyma, however, blood vessels were not found. This virtual absence of brain vascularization may be unique to hynobiid salamanders among vertebrates.
Key words. Brain; vascularization; salamander; amphibia.

The vertebrate brain demands a high oxygen supply. Indeed, the brain is generally supplied with abundant blood vessels[2,3]. To our knowledge, no vertebrates have been reported to lack blood vessels in the brain parenchyma. In hynobiid salamanders, however, we report here that the bulk of the brain parenchyma is not vascularized. This is an unusual situation and may mean that the general concept that the vertebrate brain must be intensely vascularized for its continuous and complicated activities must be seriously questioned.

Materials and methods. Mature individuals of hynobiid salamanders, *Hynobius nebulosus, Hynobius naevius,* and *Hynobius kimurai* were studied. *H. nebulosus* lives in hills and breeds in stagnant ponds. *H. naevius* and *H. kimurai* live in mountains and breed in rapidly flowing brooks. They were collected in the breeding season (winter and early spring) from their breeding sites in Shimane Prefecture, in western Japan. *H. nebulosus* measured about 9 cm, *H. naevius* about 14 cm, and *H. kimurai* about 17 cm in total length. The brains of these three salamanders were fixed in Bouin's solution and embedded in paraffin. They were serially sectioned transversely at 7 μm and stained with paraldehyde fuchsin and Masson-Goldner's method.

In *H. nebulosus* and *H. kimurai,* India ink was perfused from the heart after heparinization. In some individuals, the perfused brains were immersed in physiological saline and the meningeal surface was observed under a dissecting microscope. In other individuals, the perfused brains were fixed in Bouin's solution and embedded in paraffin. They were serially sectioned at 10 μm and observed without staining.

In *H. nebulosus,* the cerebral hemisphere, the diencephalon, the mesencephalon, and the medulla oblongata were fixed with glutaraldehyde and osmium tetroxide, and embedded in Epon. Semithin sections were stained with toluidine blue and ultrathin sections were stained with uranyl acetate and lead citrate.

For comparison, the brain of the Japanese newt, *Cynops pyrrhogaster,* was studied with routine light microscopical histology. This species was chosen for comparison because it is about the same size as *H. nebulosus* and lives in the same habitat as *H. nebulosus*[4]. In addition, the brains of the congo eel, *Amphiuma tridactylum,* and the lesser siren, *Siren intermedia,* were studied histologically, although these urodeles are much larger than hynobiid salamanders.

Results. Paraffin sections. In the brains of hynobiid salamanders, blood vessels are found 1) in the meninx covering the regular brain parenchyma, the median eminence, the paraphysis, and the choroid plexus, 2) in the small neural lobe (pars nervosa), and 3) in the olfactory bulb (fig. 1) and an adjacent small part of the cerebral hemisphere. Ependymal cells of the paraphysis and the choroid plexus are densely covered with blood vessels, some of which appear sinus-like. However, no blood vessels are found in the bulk (caudal five-sixths) of the cerebral hemisphere, the diencephalon, the mesencephalon, the metencephalon, and the medulla oblongata.

The paraphysis and the choroid plexus are large in hynobiid salamanders as in other urodele groups[5]. The telencephalic choroid plexus is located in the lateral ventricle except for its rostral extremity. The diencephalic choroid plexus is located not only in the third ventricle, but also in the mesencephalic ventricle except for its caudal extremity. The myelencephalic choroid plexus covers the fourth ventricle except for the most caudal part, which is covered with the simple posterior tela. In *Cynops pyrrhogaster, Amphiuma tridactylum,* and *Siren intermedia,* the brain parenchyma is well vascularized.

The thickness between the ventricular surface and the meningeal surface of the lateral pallium of *H. nebulosus, H. naevius,* and *H. kimurai* measures about 250 μm, 340 μm, and 390 μm, respectively. In *C. pyrrhogaster,* the thickness of the corresponding region measures about 400 μm. In the living state, the parenchymal wall must be slightly thicker than these values measured on paraffin section.

Perfusion studies. Under a dissecting microscope, a capillary net was seen on the meningeal surface of the brain. Although the shape of meshes of the net is variable, they measure approximately 50–100 μm in size. The sections of the India ink-perfused brain were carefully observed for blood vessels, but the results were the same as those with routine histology (fig. 2).

Epon sections. Both semithin and ultrathin sections were scruti-

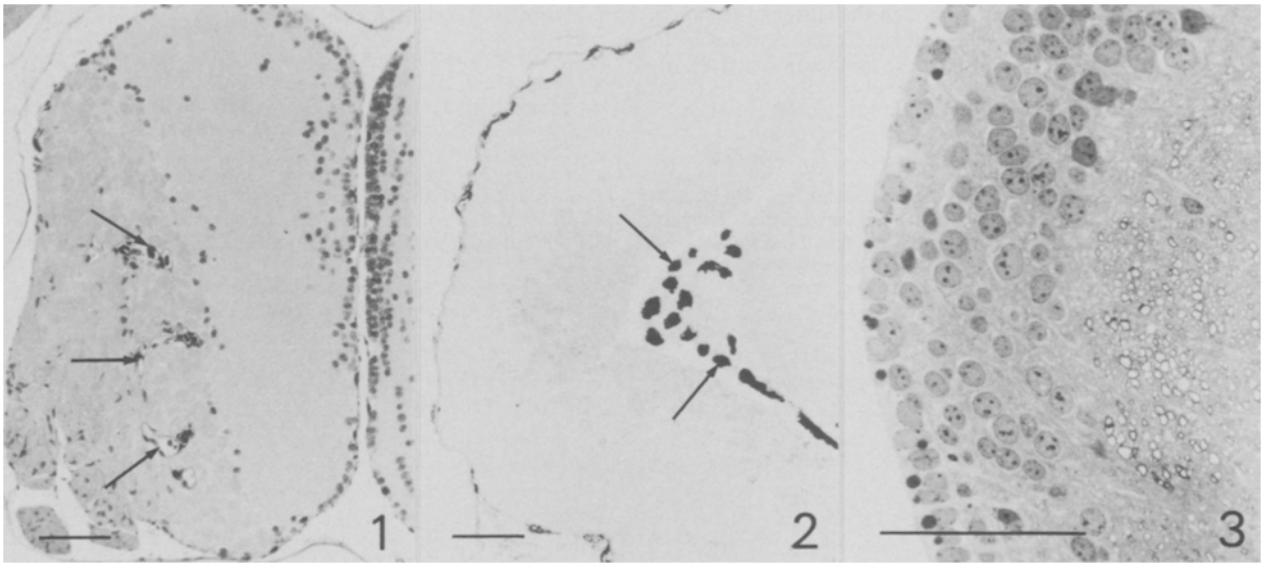

Figure 1. A transverse section of the rostral part of the telencephalon of *H. nebulosus*. Blood vessels (arrows) are seen in the olfactory bulb (left), but not in the cerebral hemisphere (right). Paraldehyde fuchsin and Masson-Goldner's staining. Scale bar 100 µm.

Figure 2. A transverse section of the cerebral hemisphere of *H. nebulosus* perfused with India ink. Note India ink-filled blood vessels (arrows) in the choroid plexus. Black structures in the meninx are partly collapsed blood vessels and partly melanophores. Tiny dots in the parenchyma are lipofuscin pigments. Blood vessels are not seen in the parenchyma. Scale bar 100 µm.

Figure 3. A transverse section of the mesencephalic tegmentum of *H. nebulosus*. Ependymal cells with lipid droplets are seen on the left and myelinated fibers on the right. Blood vessels are not found. Toluidine blue staining. Scale bar 100 µm.

nized for blood vessels with a light and an electron microscope, respectively. However, no blood vessels were found in the brain parenchyma (fig. 3).

Discussion. The brains of hynobiid salamanders (Hynobiidae) have been studied by a few authors[6,7], but in their studies nothing was mentioned about the presence or absence of parenchymal blood vessels. The present study showed for the first time that in hynobiid salamanders the bulk of the brain parenchyma is not vascularized. The most rostral small part of the cerebral hemisphere contains blood vessels even in these salamanders, but these vessels probably belong to the vasculature of the olfactory bulb.

Urodeles such as *Cryptobranchus* (Cryptobranchidae), *Ambystoma* (Ambystomidae), *Triturus* (Salamandridae), *Plethodon* (Plethodontidae), and *Necturus* ((Proteidae) are known to possess parenchymal blood vessels in the brain[8,9]. The present study showed that the brain parenchyma of *Cynops* (Salamandridae), *Amphiuma* (Amphiumidae), and *Siren* (Sirenidae) are well vascularized. Thus, all urodele families other than Hynobiidae possess a vascularized brain. The Japanese giant salamander, *Andrias japonicus*, which belongs to the same suborder (Cryptobranchoidea) as hynobiid salamanders, possesses a vascularized brain even as larvae[10]. In the lamprey, the flat spinal cord lacks parenchymal vascularization, but the brain itself contains parenchymal blood vessels[8]. The hynobiid salamanders are the only vertebrate group so far known to lack blood vessels in the bulk of the brain parenchyma.

In the brains of hynobiid salamanders, oxygen required for neuronal activity may be supplied by diffusion from meningeal blood vessels. In these animals, the choroid plexus and the paraphysis are well developed and are densely covered with blood vessels. The cerebrospinal fluid probably secreted and absorbed by these structures may represent another source of oxygen and an exit site for carbon dioxide. Herrick[11] also suggested that the choroid plexus may supply oxygen to the brain environment in sluggish animals such as salamanders whose blood is apparently poorly aerated. In hynobiid salamanders, the parenchymal wall of the brain is relatively thin. This fact favors the possibility of diffusion of oxygen from meningeal blood vessels and/or cerebrospinal fluid.

Hynobiid salamanders are distributed mainly in northeastern Asia[12], and appear to be adapted to a rather cold climate. Temperatures of their breeding ponds and brooks are about 7–8 °C[13]. At these low temperatures, the oxygen content in the water and the body fluid must be relatively high. In the terrestrial phase, they forage for food on the forest floor on rainy days and at night, and they are inactive in summer[14]. Apparently, their oxygen consumption rate is relatively low. These ecological features may also be related to the absence of parenchymal vascularization of the brain.

The 'thin parenchymal wall hypothesis' and the 'cold adaptation hypothesis' may together explain the absence of parenchymal vascularization of the brain of hynobiid salamanders. However, the results on *C. pyrrhogaster* are somewhat controversial with respect to these hypotheses. The parenchymal wall of the brain of *C. pyrrhogaster* is slightly thicker than that of *H. nebulosus*, but it is similar to that of *H. kimurai* in thickness. *C. pyrrhogaster* is more aquatic and apparently more resistant to environmental pollution than *H. nebulosus*, but they still live in the same habitat. Nevertheless, the brain of *C. pyrrhogaster* is vascularized and the brains of hynobiid salamanders are not. Apparently, these observations do not necessarily support either the 'thin parenchymal wall hypothesis' or the 'cold adaptation hypothesis'.

It is generally believed that in the 'protovertebrate' stage the brain is vascularized only on the surface (meninx), and during evolution meningeal blood vessels penetrate into the brain parenchyma either as loop-type capillaries (lamprey, salamanders, some lizards, and marsupials) or as mesh-type capillaries (other vertebrates)[2]. The 'protovertebrate' condition is represented by the present day amphioxus (Protochordata). It is surprising that such a condition is also observed in hynobiid salamanders. At any rate, they are not primitive vertebrates, although they are the most primitive urodeles[15]. The present study shows that the brain can function without parenchymal vascularization even in

a rather advanced vertebrate, although the reasons for the absence of parenchymal vascularization and the mechanism by which gas exchange is achieved have not been satisfactorily elucidated at present.

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Transport of a low molecular weight extracellular esterase into membrane vesicles of *Candida lipolytica*

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Summary. The low mol. wt extracellular esterase of *Candida lipolytica* is actively transported into membrane vesicles. In the absence of metabolic energy, a proton gradient can drive the transport process. The transport system does not accumulate the enzyme at peak levels due to the presence of a leak pathway.
Key words. Candida lipolytica; peptide; active transport proton gradient; leak pathway.

Isolated membrane vesicles from fungal and bacterial sources are used extensively to study transport at the cellular level, as they appear to retain the biological properties of membranes in intact cells[1]. The dependence of peptide transport on energy sources has been demonstrated not only for simple peptides[2,3], but also for oligopeptides[4–6]. The addition of energy uncouplers has been shown to prevent the uptake of peptides[7].

Candida lipolytica produces a low mol. wt (5700 ± 100) esterase which can be detected intra- and extracellularly. This esterase accounts in part for the observed lipolytic activity of the cells and culture medium[8]. Previous studies on the transport of this enzyme into membrane vesicles, prepared from young cultures of *Candida lipolytica*, showed that initially there was binding to the membranes followed by translocation into the vesicles[9]. This translocation process, which lead to a transport overshoot peak, required metabolic energy. Binding to the membrane vesicles was differentiated from transport into the vesicles by sonication and nystatin treatment, both of which disrupted the membrane vesicles. Both treatments abolished transport into the vesicles but not binding[9]. This paper shows that an alternative source of energy, a proton gradient, maintains the active transport of this esterase in the absence of metabolic energy.

Materials and Methods. Candida lipolytica (CMI 93743) was obtained from the Commonwealth Mycological Institute, Kew, Surrey and *Trichoderma viride* from the Central Bureau, UAST, Schimmel Cultures, Casterstraat I Baarn, The Netherlands. The snail gut juice of *Helix pomatia* was purchased from Pharmindustrie (l'Industrie Biologique Française).

Other chemicals and reagents were obtained from BDH and SIGMA and as far as possible, ANALAR grade reagents were used.

Preparation of radioactive esterase. *C. lipolytica* was grown in batch cultures in the modified medium of Fukumoto et al.[10] containing [^{14}C] glycine (10 μCi/100 ml of medium). Growth conditions were as previously described[8]. Extracellular proteins in the cell-free medium were precipitated, without prior concentration, by $(NH_4)_2SO_4$. The precipitate was dissolved, filtered through a Sephadex G-100 column and the active esterase fractions were pooled and concentrated as described earlier[8]. The final protein concentration was 0.2 mg/ml.

Preparation of membrane vesicles of *C. lipolytica*. Snail gut juice of *Helix pomatia* was used without any further purification or dilution. Lytic enzyme was obtained by growing *T. viride* in batch cultures on a rotary shaker at 28 °C for 10 days. The medium contained 3 g glucose; 1.4 g $(NH_4)_2SO_4$; 2 g KH_2PO_4; 0.3 g $MgSO_4 \cdot 7H_2O$; 0.3 g $CaCl_2$; 0.3 g urea; 1.5 g *C. lipolytica* cell walls and 1 ml of trace metal solution per l. The trace metal solution contained 500 mg $FeSO_4$; 150 mg $MnSO_4$; 167 mg $ZnCl_2$; 200 mg $CoCl_2$ and 1 ml 19% HCl per 100 ml. The mycelium was harvested by centrifugation at 5000 × g. The cell-free medium was filtered and freeze-dried. This enzyme was used without further purification.

To about 1.5 g of 24-h-old *C. lipolytica* cells was added 1.4 ml of 0.1 M EDTA and 0.05 ml of 2-mercaptoethanol. Distilled water was added to a final volume of 5 ml and the suspension incubated for 90 min at 35 °C. After washing, 1 g of the cells was suspended in 10 ml 0.1 M KH_2PO_4 buffer, pH 6.4, containing 1 M $MgSO_4$ as an osmotic stabilizer. The freeze-dried lytic enzyme (200 mg), and 0.05 ml of undiluted *Helix pomatia* gut juice were added and the incubation was continued at 35 °C with minimal shaking. Protoplast formation was complete within 3–4 h of incubation. Protoplasts were purified by differential centrifugation at 4 °C.

The suspension of protoplasts was centrifuged at 10,000 × g and approximately 0.5 g of the resulting pellet was suspended in 20 ml distilled water at room temperature for 30 min. In the absence of an osmotic stabilizer, the protoplasts of *C. lipolytica* lysed immediately. Buffer (0.1 M KH_2PO_4, pH 6.4 containing 10 mM $MgSO_4$) was added and the suspension was centrifuged at low speed and then at 14,000 × g to sediment membrane vesicles. The membrane vesicles were washed three times with the same buffer. Vesicles were resuspended in the 0.1M KH_2PO_4 buffer or