GLIA 56:699–708 (2008)

All Vertebrates Started Out with a Glial Blood-Brain Barrier 4–500 Million Years Ago

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KEY WORDS
fish; evolution; brain blood vessels; horseradish peroxidase; tracer; astrocyte; basal lamina

ABSTRACT
All extant vertebrates have a blood-brain barrier (BBB), a specialized layer of cells that controls molecular traffic between blood and brain, and contributes to the regulation (homeostasis) of the brain microenvironment. Such homeostasis is critical for the stable function of synapses and neural networks. The barrier is formed by vascular endothelial cells in most groups, but by perivascular glial cells (astrocytes) in elasmobranch fish (sharks, skates, and rays). It has been unclear which is the ancestral form, but this information is important, as it could offer insights into the roles of the endothelium and perivascular glia in the modern mammalian BBB. We have used electron microscopic techniques to examine three further ancient fish groups, with intravascular horseradish peroxidase as permeability tracer. We find that in bichir and lungfish the barrier is formed by brain endothelial cells, while in sturgeon it is formed by a complex perivascular glial sheath, but with no detectable tight junctions. From their BBB pattern, and position on the vertebrate family tree, we conclude that the ancestral vertebrate had a glial BBB. This means that an endothelial barrier would have arisen independently several times during evolution, and implies that an endothelial barrier gave strong selective advantage. The selective advantage may derive partly from greater separation of function between endothelium and astrocytic glia. There are important implications for the development, physiology, and pathology of the mammalian BBB, and for the roles of endothelium and glia in CNS barrier layers.

INTRODUCTION

The great American physiologist Homer W. Smith used careful study of kidney tubules of different vertebrate groups together with the geological context to show how adaptations of a single organ could have contributed to human evolution (Smith, 1953; Vize and Smith, 2004). Natural selection led to adaptations of the kidney in response to the move from salt to fresh water, and from thence to the land or back to sea water following climatic and geological change. In this study we have applied similar principles to examination of the blood-brain barrier (BBB).

The mammalian BBB is formed by the brain capillary endothelium under the inductive influence of surrounding cells especially the closely-apposed endfeet of astrocytic glia (Abbott et al., 2006). The barrier helps establish a specialized microenvironment for optimal neuronal function (Abbott, 1992). Many neuropathologies involve BBB dysfunction, and in some cases (tumors, epilepsy), compensatory changes in the glial cells are observed, so they act as a "second line of defense" (Abbott et al., 2006; Bronger et al., 2005; Marroni et al., 2003). This study of the evolution of the vertebrate BBB gives insights into the origin of the division of labor between endothelial cells and astrocytes in the mammalian BBB, and helps to explain not only features of the embryonic development and adult organization of the mammalian brain, but also changes occurring in human pathology.

Among both invertebrates and vertebrates, neural tissue has become more centralized, larger, and more complex during the course of evolution (Abbott et al., 1986; Nieuwenhuys et al., 1998; Nieuwenhuys, 2002), allowing more effective coordination of body function. This clearly gives selective advantage in Darwinian terms. However, larger neural masses require more efficient ways of delivering nutrients and clearing waste products, hence the extensive blood vessel network supplying the brains of advanced invertebrates such as decapod crustaceans (crabs, lobsters) and cephalopod mollusks (octopus, squid, cuttlefish) (Abbott et al., 1986; Abbott, 1992). All vertebrates have intracerebral blood vessels, continuing this pattern.

A further selective advantage is given by development of a BBB (Abbott, 1992; Cserr and Bundgaard, 1984). The barrier helps to keep out toxic agents, controls molecular entry and efflux by specific transport systems, and contributes to regulation (homeostasis) of the brain microenvironment (Abbott, 2005; Begley and Brightman, 2003). Selective advantage comes from the resulting more efficient neural signaling. In invertebrates the endothelial lining of the blood vessels is often incomplete or leaky, and the barrier where present is formed by perivascular or boundary glial cells (Abbott et al., 1986;

This article contains supplementary material available via the Internet at http://www.interscience.wiley.com/jpages/0894-1491/suppmat.

Grant sponsor: Danish Natural Science Research Council.

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Received 15 June 2007; Accepted 21 December 2007

DOI 10.1002/glia.20642

Published online 13 March 2008 in Wiley InterScience (www.interscience.wiley.com).

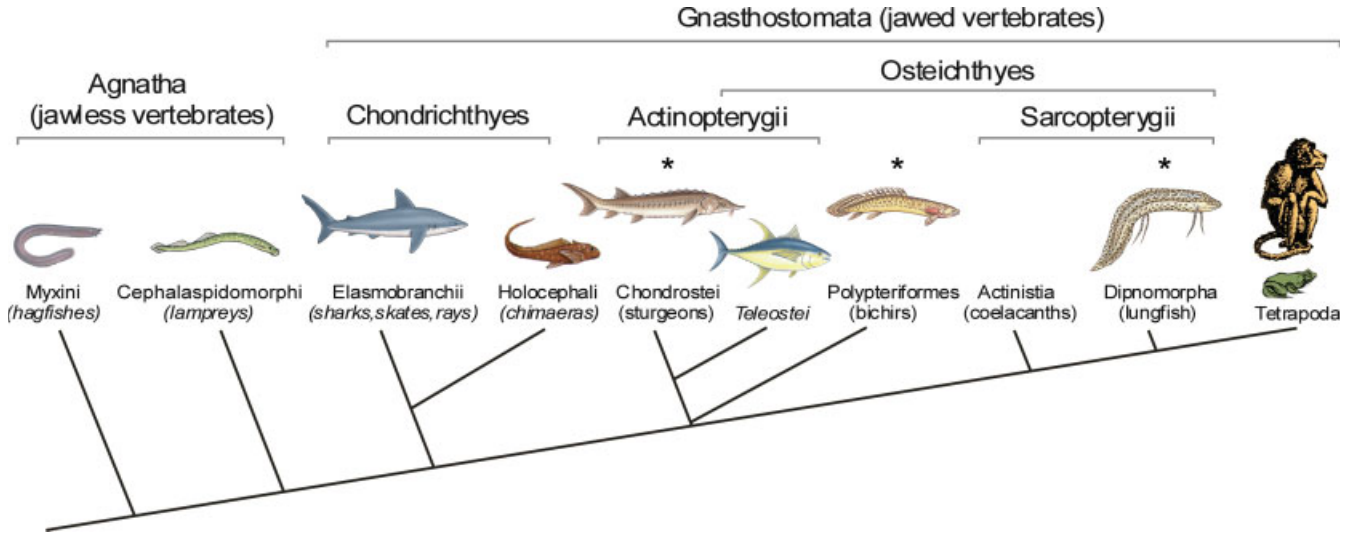

Fig. 1. Cladogram showing the evolution of major living fish groups 500–350 M years ago and origin of tetrapods, based on Janvier 1996; Venkatesh et al., 2001; Hickman et al., 2004. Of the fish groups previously studied (italics), elasmobranchs have a glial blood-brain barrier, while hagfish, lamprey, chimaera, teleosts (and tetrapods) have an endothelial barrier. The further archaic fish groups examined in this study are asterisked: sturgeon, bichir and lungfish. (Fish illustrations from Hickman et al. (2004) by permission of McGraw Hill).

Abbott, 1992). In most vertebrates including tetrapods (amphibia, reptiles, birds, and mammals), the barrier is formed by the brain microvessel endothelium (Cserr and Bundgaard, 1984), suggesting that once the endothelium becomes tight enough it can take on the barrier role.

Several fish groups have been previously investigated, shown in italics on the cladistic diagram, Fig. 1 (Hickman et al., 2004; Janvier, 1996). Sharks, skates, and rays (Chondrichthyes: Elasmobranchii) have a glial barrier (Brightman et al., 1971; Bundgaard and Cserr, 1981b), while three other extant fish groups with some archaic features, hagfish (Myxini) (Bundgaard and Cserr, 1981a), lampreys (Cephalaspidomorphi) (Bundgaard, 1982b), and chimaeras (Chondrichthyes: Holocephali) (Bundgaard, 1982a) as well as modern ray-finned bony fish (Actinopterygii: Neopterygii, teleosts) (Brightman and Reese, 1969) have an endothelial barrier. Hence either the ancestral vertebrates(s) had an endothelial barrier which was lost in elasmobranchs (Cserr and Bundgaard, 1984), or the ancestral form was a glial barrier, retained in elasmobranchs but replaced by an endothelial barrier in other groups (Abbott, 1992). If the ancestral barrier was glial, it could help explain the residual ability of glia to form barriers in the mammalian nervous system, in development, adult organization, and pathology (Abbott, 1992).

Abbreviations

BBB	blood-brain barrier
CP	choroid plexus
CNS	central nervous system
HRP	horseradish peroxidase
K+	potassium ion
Myr	millions of years
MS222	methane sulfonic acid salt of ethyl-m-amino-benzoate
RPE	retinal pigment epithelium.

We predicted that if a glial barrier is the ancestral form, it should be present in at least some of the most archaic fish still living. In this study, we chose three further fish groups occupying key positions on the evolutionary cladistic diagram (Fig. 1, and Fig. 7 in Venkatesh et al., 2001), to establish whether the vertebrate BBB was originally glial or endothelial. Sturgeons (Chondrostei) and Bichirs (Cladistia: Polypteriformes) are members of ancient families of ray-finned fish (Actinopterygii), while lungfish belong to "lobe-finned fish" (Sarcopterygii: Dipnomorpha) (Janvier, 1996). We used electron microscopy to obtain high-resolution images of the cerebral blood vessels, and intravascular horseradish peroxidase (HRP) as tracer to identify barrier sites (Brightman and Reese, 1969).

MATERIALS AND METHODS

Experimental Animals

Fourteen sturgeons (Russian *Acipenser güldenstädtii*; 14–65 g; Siberian *Acipenser baerii*; 42–115 g), six bichirs (*Polypterus senegalus*; 21–55 g) and two African lungfish (*Protopterus annectens*; 91 and 108 g) were examined. They were obtained commercially from a local dealer in aquarium fishes and kept in aerated standing tap water at room temperature. Animals were anaesthetized by immersion in tap water containing MS 222 (0.25 g/L; methane sulfonic acid salt of ethyl-m-amino-benzoate, Sandoz, Copenhagen, Denmark), with anesthesia maintained by flow of oxygenated MS222 solution (0.1 g/L) over the gills (~25 mL/min). The anesthetic and surgical procedures complied with Danish legislation, and were approved by the controlling body under the Ministry of Justice.

Electron Microscopy

The fixative was 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Most brains were fixed by perfusion via a catheter in the heart ventricle after large cardiac veins had been cut to permit outflow of fixative. Some sturgeon and bichir brains were fixed by *in situ* immersion overnight after removing overlying cartilaginous skull and muscles. Small (<0.5 mm) tissue blocks were then cut from the telencephalon and optic tectum (selecting dorsal parts from *in situ* immersion fixed material), and washed in buffer.

Tracer Experiments

Horseradish peroxidase (HRP; Sigma, type II, Copenhagen, Denmark) was used as electron microscopic tracer in some animals (Karnovsky, 1968). A freshly prepared solution of HRP in phosphate buffered saline (200 mg/mL) was slowly injected into either the posterior cardinal vein or the heart ventricle (0.25 mL/100 g animal). The time between tracer injection and start of fixation (circulation time) was in the range 5–23 min. After fixation blocks of brain tissue were incubated for peroxidase activity (Abbott and Bundgaard, 1992). Samples from noninjected animals were included as controls for endogenous peroxidases.

Further Preparation for Electron Microscopy

After additional buffer rinses all tissue blocks were postfixed for 2 h in 2% OsO_4 in 0.1 M cacodylate buffer, then stained in 2% aqueous uranyl acetate solution at 60°C for 15 h. After dehydration in graded ethanol followed by propylene oxide, the blocks were embedded in Epon. 1-2 μm light microscopic sections were stained with Toluidine blue for orientation and to establish tissue structure, and unstained sections were used to localize HRP reaction product. Thin sections of selected areas were examined in a Zeiss 10B electron microscope operated at 60 kV.

RESULTS

In each species examined, brain capillaries from telencephalon and optic tectum showed similar light microscopic and ultrastructural characteristics. Variation in HRP circulation times was not reflected in variation in tracer distribution. Among sturgeons, there were no observable differences between *Acipenser baerii* and *A. güldenstädtii*. Endogenous peroxidase was observed only in erythrocytes (Supplementary figures, Figs. S1A,S1B).

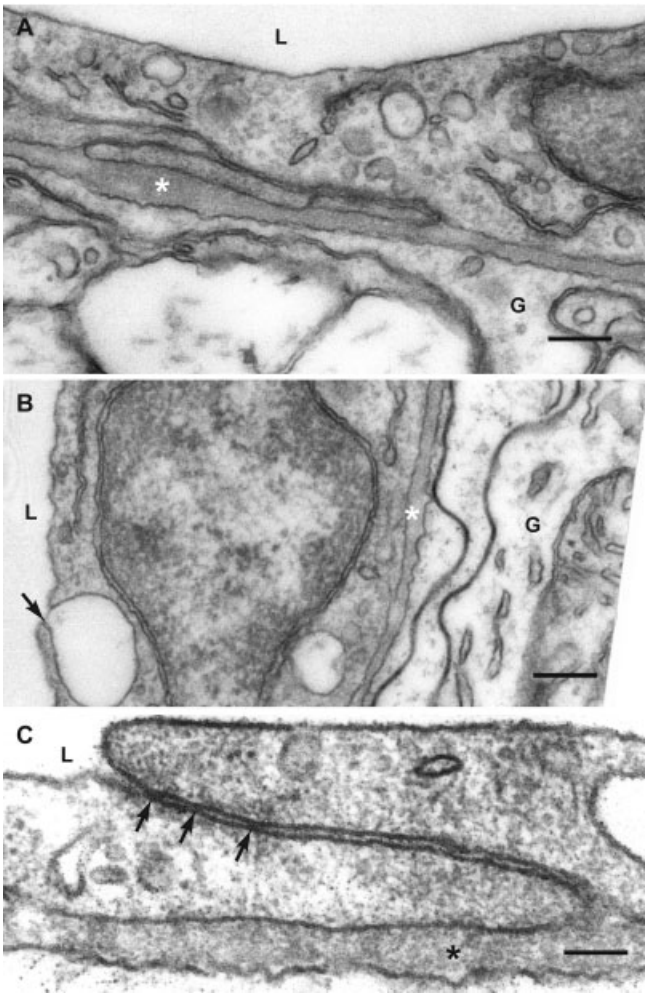

Fig. 2. Sturgeon brain. Electron micrographs after perfusion fixation. (**A**, **B**) Segments of endothelia from brain capillaries, showing vessel lumen (L). Note vesicular membrane profiles in the endothelial cytoplasm. Larger membrane-bound cavities as in (B) were regularly encountered. The arrow marks such a structure with an opening to the luminal surface of the cell. The endothelial basal lamina (asterisk) is 40–80 nm thick. Characteristic flattened glial cells (G) always separate neuronal processes from endothelium. (**C**) Segment of capillary wall, lumen (L) to left, including an endothelial intercellular junction. Adjacent cell membranes form three punctate contacts (arrows). Scale bars A, B, 0.2 μm; C, 0.1 μm. For fuller frame views of Figs. 2A,B, see Supplementary figures Figs. S2A,S2B.

In Brain Microvessels of Sturgeon, Perivascular Glia form a Diffusion Barrier

In sturgeon, the brain endothelium was continuous, with conspicuous caveolae and vesicular profiles (diameter 60–80 nm) (Fig. 2, S2) and occasional larger diameter invaginations (200–300 nm) closed by a thin diaphragm (Figs. 2B,S2B). Neighboring endothelial cells were connected by tight junction-like contacts, typically with 2–3 membrane contacts in series (Fig. 2C), overlying a 50–80 nm thick basal lamina. Typical pericytes were not observed, but the capillary wall appeared in places to contain overlapping endothelial cell processes (Fig. 3C).

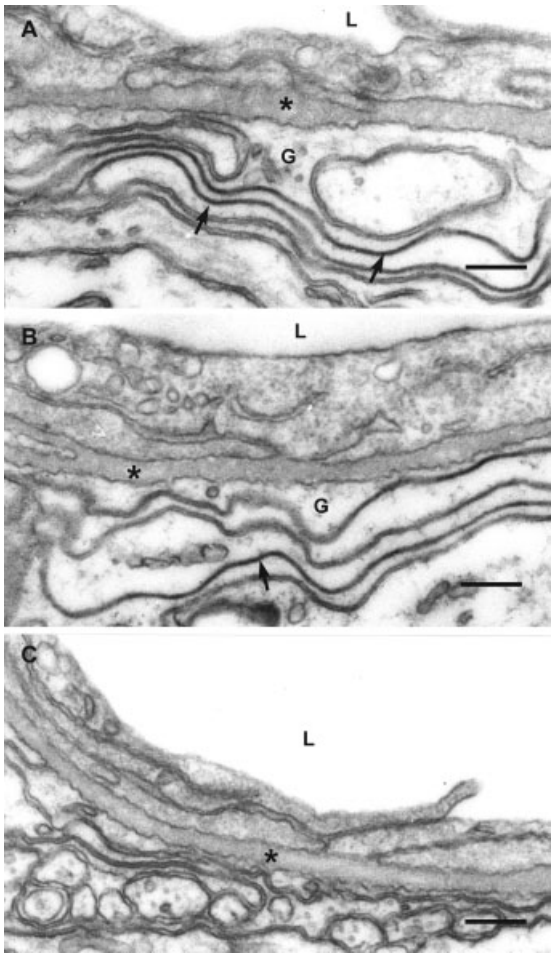

Fig. 3. Sturgeon brain. This montage of electron micrographs illustrates the complex geometry of extremely attenuated (20 nm) processes of glial cells (G) separating neural tissue from capillary endothelium; lumen (L) at top, asterisk marks basal lamina. In (**A**) and (**B**), arrows mark adjacent parallel glial cell membranes organized as typical gap junction-like contacts. In (**C**) several fine nerve fibers/neuronal processes are seen enwrapped by glial cell processes and separated from the endothelial basal lamina. Scale bars 0.2 μm.

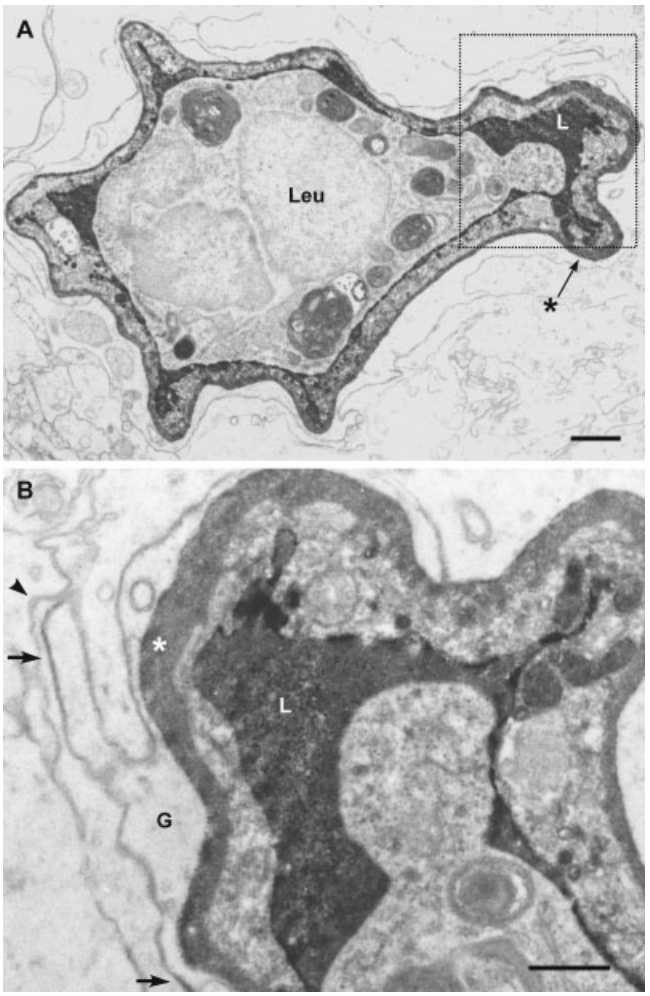

Fig. 4. Sturgeon brain fixed by immersion after HRP-injection, transverse section of capillary containing leukocyte (Leu). (**A**) The capillary lumen (L) and endothelial basal lamina (asterisk) are labeled by the electron-dense reaction product. The surrounding brain tissue appears unlabeled at this magnification. (**B**) Higher magnification view of region marked with dotted line in (A). Electron-dense material (reaction product) occurs focally (arrows) between some processes of pericapillary glial cells (G), but deeper extracellular clefts are unlabeled (arrowhead). Scale bars A, 1 μm; B, 0.5 μm. For fuller frame views of Fig. 4B, see Fig. S3.

Capillaries were surrounded by multiple layers of glial cells, often extremely attenuated (10 nm), arranged in a complex geometry (Figs. 2A, B, S2A, S2B, and 3). The diffusion distance from endothelial basal lamina to nerve cells could rarely be determined because sooner or later the plane of the cleft approached the plane of the section and consequently was unidentifiable. For this reason attempts to follow the diffusion pathway from endothelium to nerve cells usually stopped after 10–15 μm. The shortest pathway was determined in four cases only. The distances were 10, 11, 12, and 22 μm. Tight junctions between glial cells were not identified, but neighboring cell membranes often followed a parallel course in close proximity—an arrangement typical of gap junctions.

Intravenously injected HRP permeated the cerebral capillary endothelium, clearly labeling the basal lamina with reaction product (Figs. S1C, S1D; Fig. 4 and Fig. S3; Fig. 5, and Fig. S4; Fig. 6). In light microscope sections, HRP reaction product was not detected in brain parenchyma, although it labeled extravascular cells outside the nervous system (Fig. S1D). At the EM level, tracer was detected in segments of inter-endothelial junctional zones and within vesicular profiles, including those opening to either luminal or abluminal surfaces. No abrupt stoppage of tracer penetration was observed on the brain side of the basal lamina. However, electron-dense material in low concentration was observed focally between the perivascular glial cells, but not among underlying neuronal processes (Figs. 4, 5, and 6; Figs. S3 and S4). The results indicate that the perivascular glial layer is the major diffusion barrier between blood and brain in the sturgeon.

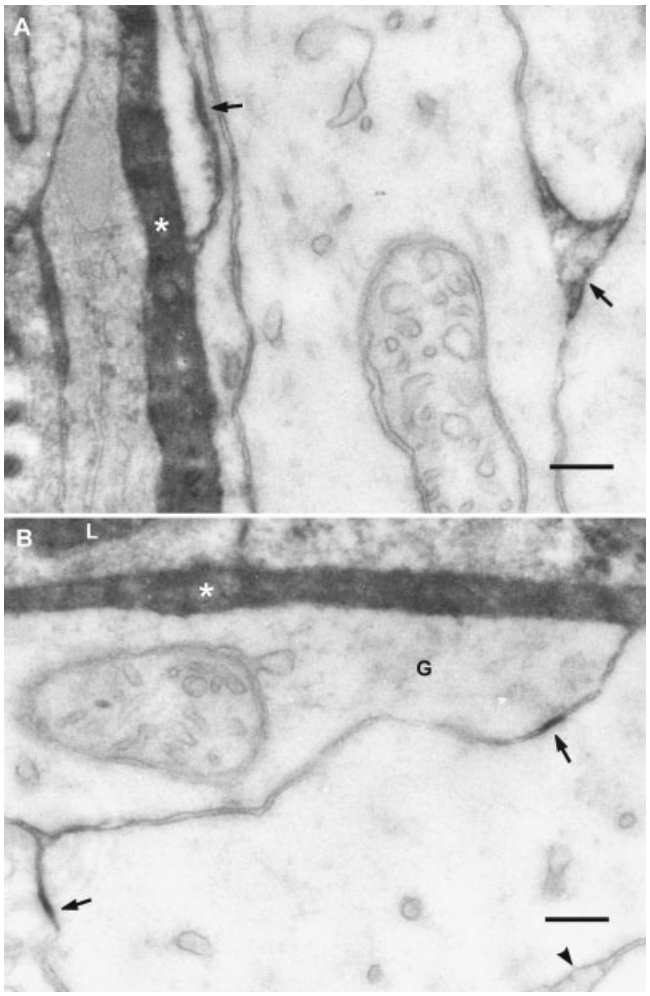

Fig. 5. Sturgeon brain, fixed by immersion after HRP-injection. In (**A**) capillary lumen is off to left, in (**B**) it is shown at top (L); endothelial basal lamina (asterisk) contains reaction product. Arrows mark HRP reaction product between perivascular glial cell processes, while arrowhead (in **B**) shows absence of labeling in a deeper cleft. Scale bars 0.2 μm. For fuller frame views of Figs. 5A,B, see Figs. S4A,S4B.

Bichir and Lungfish Have an Endothelial Blood-Brain Barrier

Bichir and lungfish showed a cerebral capillary ultrastructure markedly different from sturgeon, and more like that of teleost fish (Brightman and Reese, 1969). In both groups the endothelium was continuous and more electron dense than cells of the surrounding neuropil (Figs. 7A, S5; Figs. 8A, S8).

In bichir occasional cross-sectioned brain capillaries had no detectable interendothelial cleft ("seamless capillary") (Figs. 7A, S5). Endothelial caveolae and vesicular profiles were rare. The endothelial basal lamina was thin, less than 30 nm. Elaborate endothelial junctional zones with tight junction contact points were observed (Figs. 7B,C). The layer of pericapillary glia overlying small axons and neuronal processes was incomplete (Figs. 7D,E, S6A, and S6B). There were no signs of transendothelial passage of intravenously injected HRP in either light microscopic images (not shown) or at the electron microscopic level (Figs. 7F, S7).

In lungfish (Figs. 8, S8, S9, and S10) brain endothelium, caveolae, and vesicular profiles were present (Fig. 8B) but not as frequent as in mammalian extracerebral capillary endothelium. Clefts between neighboring endothelial cells appeared sealed by elaborate tight junctions with multiple (>5; Fig. 8B) punctate membrane contacts/fusions in series. Partially surrounding the endothelial basal lamina (80–100 nm thick) was a further incomplete layer of electron-dense perivascular cells with no further basal lamina on the brain side. Zones of close contact between these cells and the abluminal basal surface of the endothelium were regularly encountered (Fig. 8C). The layer of pericapillary glial cells was incomplete, and unmyelinated nerve fibers were observed in close association with the endothelium and perivascular cells (Figs. 8B,D, and S9). HRP reaction product was seen in the capillary lumen of the perfuse-fixed brains and in intraendothelial vesicles (Figs. 8E and S10), but was not detectable on the brain side of the endothelium after 5 or 10 min circulation, at the level of either the light microscopic (not shown) or electron microscope.

DISCUSSION

Our results indicate that in bichir and lungfish brain, barrier properties are associated with the endothelium. The endothelium is continuous, with the type of tight junction-like contacts characteristic of a tight endothelium. The presence of "seamless" capillaries in bichir is also characteristic of very tight endothelia, as observed in mammalian brain (Oldendorf et al., 1977). By contrast, the layer of perivascular glial cells is incomplete and nerve fibers directly contact the endothelial basal lamina. The tracer experiments support the ultrastructural observations, showing that the brain capillary endothelium in bichir and lungfish is a diffusion barrier to HRP.

In the lungfish, the cell layer structure of the brain microvessel walls is slightly different from that of amphibian and mammals, in that no clear pericytes are seen, but electron-dense cells outside the endothelial basal lamina (perivascular cells, Fig. 8B) make close contacts with the endothelium through breaks in the basal lamina. These contacts resemble the endotheliopericytic and myoendothelial junctions seen in the walls of mammalian microvessels (Allt and Lawrenson, 2001; Rhodin, 1967) but the absence of basal lamina between the dense cells and underlying electron-lucent glial cells suggests they could be a glial cell type. The cellular interdigitation suggests a site for cell:cell communication and regulation. Further investigation would be of interest.

The two sturgeon species studied are not closely related according to genetic analysis (Birstein and De Salle, 1998); the consistency of findings between the two species hence confirms the generality of the results. The ultrastructure of the sturgeon brain capillary endothe-

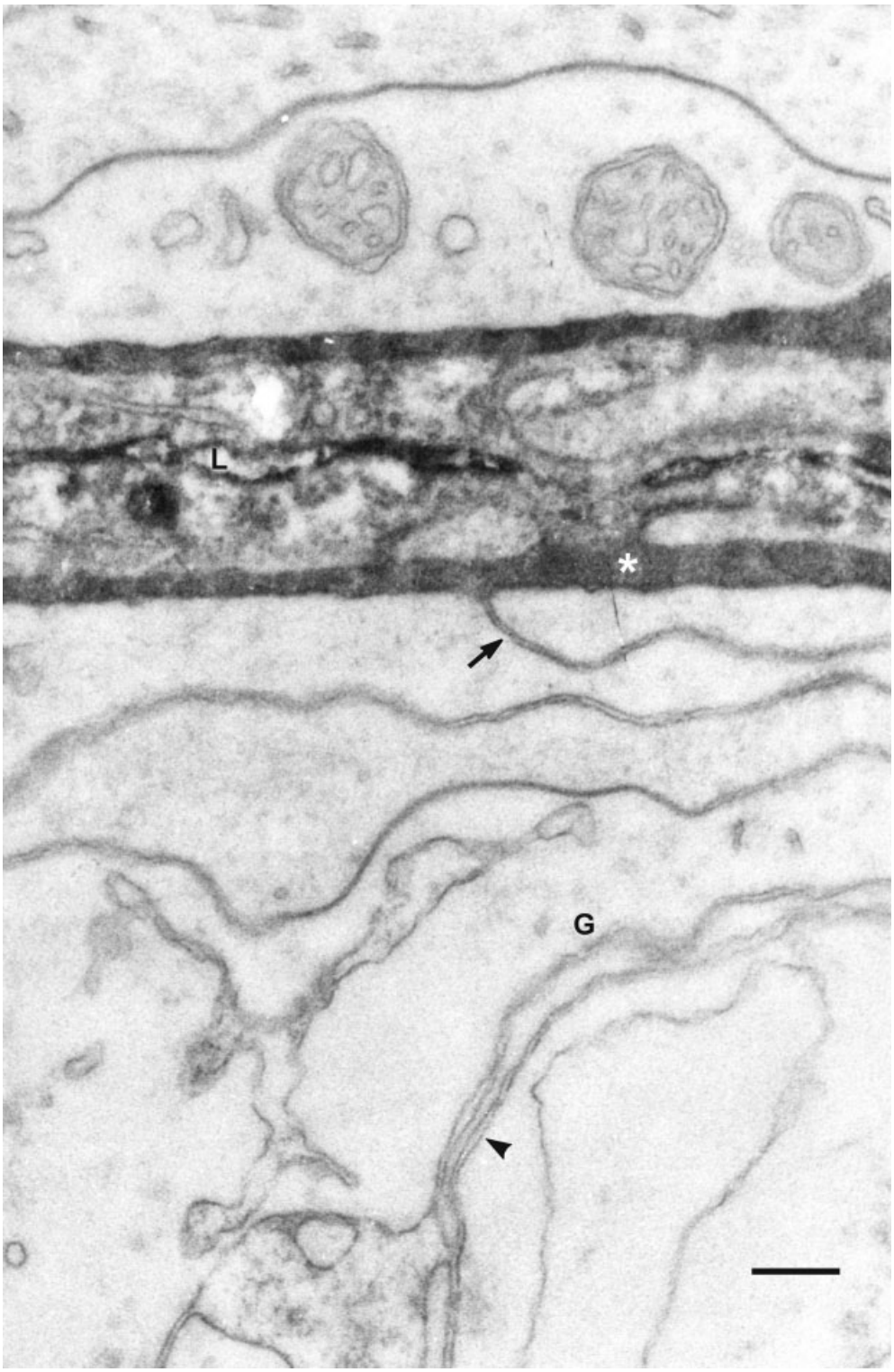

Fig. 6. Sturgeon brain fixed by immersion after HRP-injection. A longitudinally sectioned capillary with partially collapsed lumen (L) is seen in the upper part of the figure. Capillary lumen and basal lamina (asterisk) contain HRP reaction product. Arrow marks the opening of a cross sectioned cleft between perivascular glial cells. Neighboring glial cells appear not be connected by specialized cell contacts in this region, although a faint banding/periodicity can be detected. However, labeling of the extracellular space decreases with increasing distance from the basal lamina; arrowhead marks unlabeled intercellular clefts deeper in tissue. Scale bar 0.2 μm.

lium resembles that of mammalian muscle capillaries, with a conspicuously high number of caveolae and vesicular profiles, and intercellular clefts interrupted by 2–4 punctate membrane contacts. In mammalian (mouse) muscle capillaries HRP was found to permeate this type of endothelium (Williams and Wissig, 1975), although circulation times >16 min were needed to see clear extravasation. In the present study, HRP permeated sturgeon brain endothelium after 10–23 min circulation time, slower than extravasation in sharks and skates (within 1 min) (Brightman et al., 1971; Bundgaard and Cserr, 1981b); it was not possible to determine whether

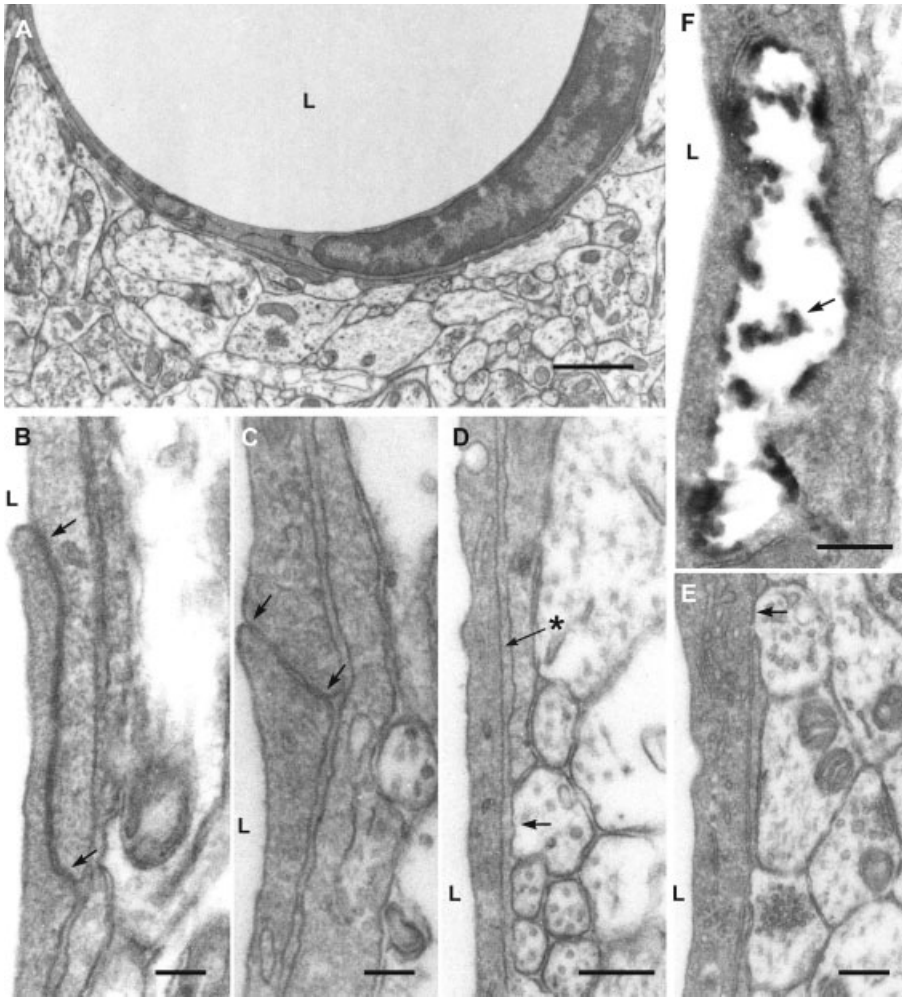

Fig. 7. Bichir brain capillary segments, showing vessel lumen (L) and electron-dense endothelial cells. (**A**) Low magnification electron micrograph of brain capillary. There was no intercellular cleft detectable in the continuous endothelium when it was followed round the whole profile in this vessel ("seamless capillary"); a segment is shown here, the full image in Supplementary Fig. 5. (**B**,**C**) Segments of brain capillary endothelium. Arrows indicate the depth of endothelial tight junctions. (**D**,**E**) Blood-brain interface. Pericapillary coverage is missing at arrows, so that neuronal process abut directly the thin basal lamina (asterisk). (**F**) Tracer distribution. Bichir fixed by perfusion 15 min after intravenous injection of HRP. Tracer has been washed out of the capillary lumen, however, a membrane-bound structure (invagination?) contains reaction product (arrow). Scale bars A, 1 μm; B, C, 0.1 μm; D–F 0.2 μm. For fuller frame views of Figs. 7A,D,E, and F, see Figs. S5, S6A, S6B, Fig. S7.

HRP permeated via vesicles or junctional clefts. HRP was not detected among neuronal processes beyond the perivascular glial layers. The complex multilayered glial sheath with gap junction-like contacts would represent an appreciable restriction to diffusional exchange of hydrophilic solutes. The average "cleft depth" of >10 μm is much greater than the ~0.5 μm reported for extracerebral continuous endothelia in amphibia and mammals (Bundgaard and Frøkjaer-Jensen, 1982); other things being equal this would result in reduction in diffusional permeability by a factor of ~20. In mammals, the brain endothelium is ~100 times less permeable to small hydrophilic solutes than that of skeletal muscle capillaries (Crone, 1986). A similar mechanical restriction may be obtained in sturgeon by a moderately restrictive endothelium in series with the multilayered glial wrapping.

The presence of a glial BBB in sturgeons (Chondrostei, Actinopterygians) and elasmobranchs (Chondrichthyes) has important implications for the evolution of the vertebrate BBB. Chondrichthyes are recognized as the most basal of living gnathostomes (jawed vertebrates), according to anatomical, palaeontological, and genetic analysis (Janvier, 1996; Kikugawa et al., 2004). Although not closely related, elasmobranchs and chondrosteans preserve many features of more archaic fish anatomy and physiology (Bone et al., 1995). Thus elasmobranches have a cartilaginous skeleton, teeth not fused to jaws and usually replaced, no swim bladder, intestine with spiral valve, claspers present in males, "placoid" scales, five to seven gill arches and gill slits in separate clefts along pharynx, open spiracle, upper jaw not fused to cranium, and a more limited repertoire of genes and signaling peptides than more modern fish groups (Bone et al., 1995; Hickman et al., 2004; Venkatesh et al. 2001). Chondrosteans have a primarily cartilaginous skeleton, ganoid scales, open spiracle, swim bladder opening to dorsal side of pharynx, spiral valve, more fin rays than ray supports, and again a limited genomic profile (Hickman et al., 2004; Venkatesh et al., 2001). We propose that a glial BBB in these groups should also be seen as an archaic feature.

As hagfish and lamprey (Agnatha, jawless fish, living representatives of the most ancient vertebrate group) and the archaic Holocephali (ratfish, *Chimaera monstrosa*) have an endothelial barrier, Cserr and Bundgaard (1984) proposed that the ancestral vertebrate had

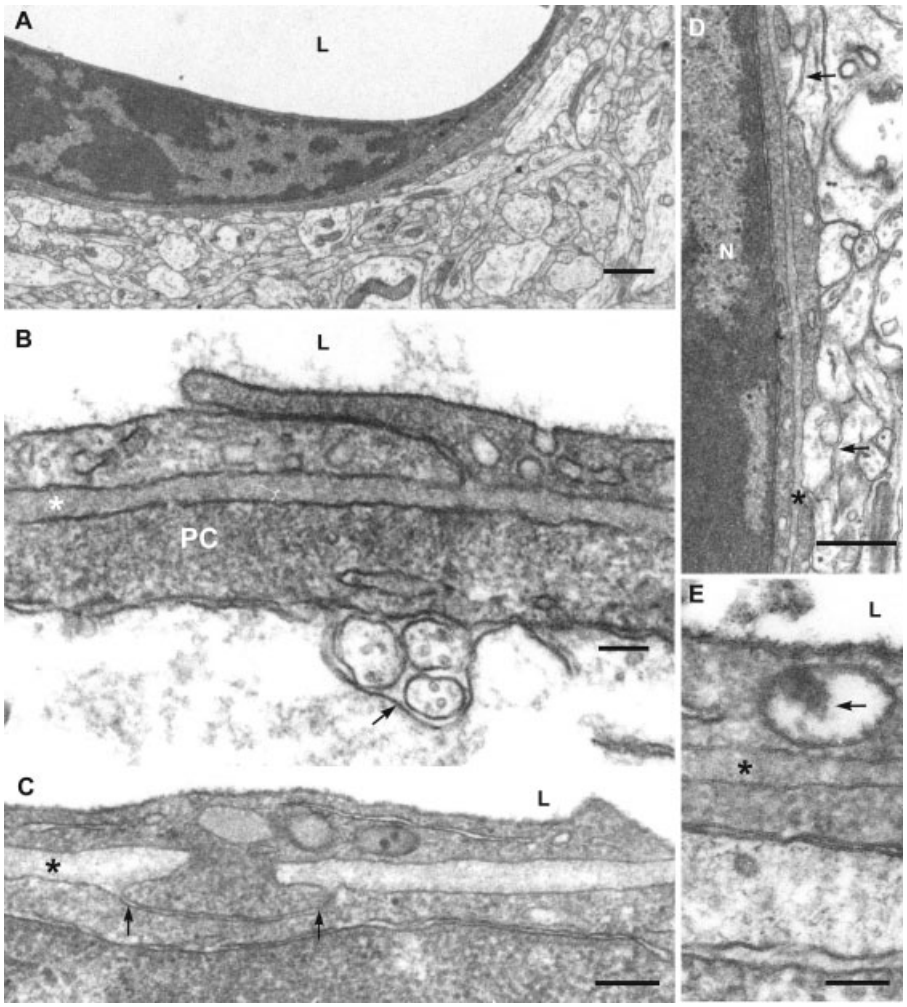

Fig. 8. Capillaries in lungfish telencephalon. Lumen (L) shown at top (A–C, E), or off to left (D); asterisks mark basal lamina. (**A**) Low magnification electron micrograph of capillary endothelium. The endothelium appears continuous. The cytoplasm of endothelium and adjacent perivascular cell is more electron dense than the cytoplasm of the surrounding neuropil. (**B**) Segment of endothelium. A cleft between endothelial cells is located in the middle. The intercellular space is interrupted by multiple contacts between adjacent cell membranes. Arrow marks three neuronal processes next to an electron-dense perivascular cell (PC). (**C**) Segment of brain capillary. An endothelial process (arrows) indents a neighboring perivascular cell. (**D**) Brain capillary, showing endothelial nucleus (N). Arrows mark neuronal processes separated from the endothelium by the basal lamina only. (**E**) Tracer distribution. Lungfish fixed by perfusion 10 min after intravenous injection of horseradish peroxidase. The electron micrograph shows a segment of capillary endothelium from optic tectum. Small amounts of electron-dense material (reaction product) remain in the capillary lumen, and reaction product adheres to the luminal surface of the endothelium. A membrane-bound structure (probably an invagination of the luminal membrane) contains reaction product (arrow). Scale bars A, 1 μm; B, E, 0.1 μm; C, 0.2 μm; D, 0.5 μm. For fuller frame views of Figs. 8A,D, and E, see Figs. S8, S9, and S10.

an endothelial BBB, with the elasmobranch glial BBB being a later and anomalous (secondary) development. Figure 9A illustrates this view. According to this scheme, the glial barrier in the sturgeon would be another secondary development.

By contrast, on the basis of a predominantly glial barrier among invertebrates and in elasmobranch fish, Abbott (1992) argued that the ancestral vertebrate barrier was glial, with an endothelial barrier evolving later. In the embryonic mammalian brain, glial barriers separate the developing nervous system from vascular elements—in the *glia limitans* formed by the endfeet of radial glia under the pia, and the ependyma lining the ventricles (Saunders et al., 1999). These barriers disappear or are modified once vessels enter the brain. Elements of a glial barrier are still seen in adult vertebrates, in the *glia limitans* of the avascular spinal cord of lamprey (Fraher and Cheong, 1995), and in certain mammalian ependymal derivatives [ependymoglia and tanycytes especially in circumventricular organs, choroid plexus (CP), and retinal pigment epithelium (RPE)] (Abbott et al., 1986; Reichenbach and Wolburg, 2004). In these specialized ependymal derivatives of mammals, a physical barrier resulting from intercellular tight junctions can be detected (Rodriguez et al., 2005; Vorbrodt and Dobrogowska, 2003), and specific transporters regulating transcellular traffic are well documented in CP and RPE (Cunha-Vaz, 2004; Redzic et al., 2005).

Tight junctions between astrocytes have been observed in the critical growth/differentiation zone of

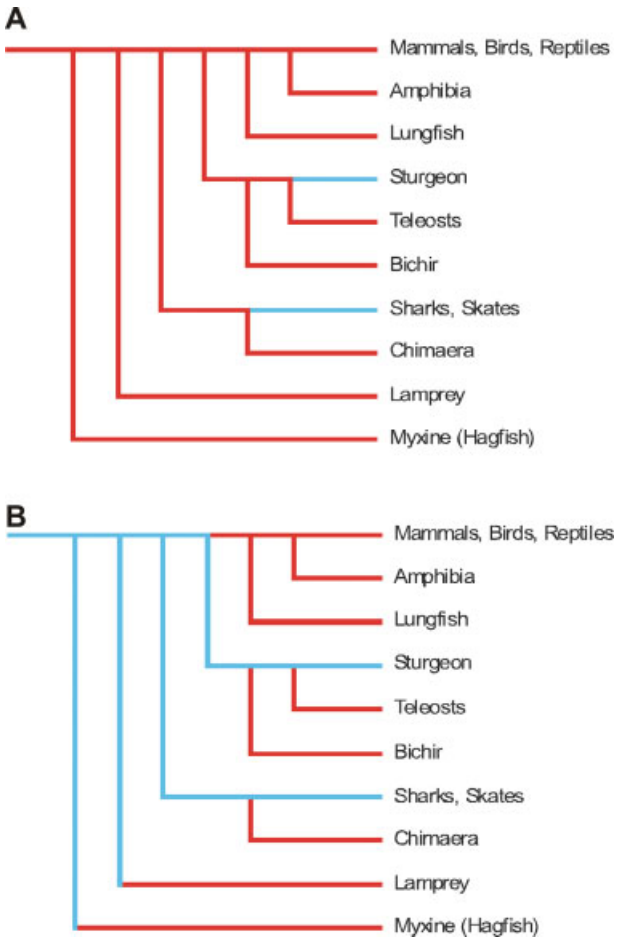

Fig. 9. Alternative models of the phylogeny of the vertebrate BBB. The phylogenetic relationships of gnathostomes (jawed vertebrates) are based on Venkatesh et al. (2001). Blue lines indicate a glial BBB, red lines endothelial. (**A**) The demonstration of an endothelial barrier in lamprey, hagfish and chimaera as well as in all advanced vertebrates led to the proposal that the ancestral vertebrate had an endothelial barrier (Cserr and Bundgaard, 1984). In this scheme, the glial barrier in elasmobranchs and sturgeon would be unusual secondary developments. (**B**) By contrast, the present study supports the view that the ancestral vertebrate had a glial barrier which has been replaced by an endothelial barrier several times in evolution (Abbott, 1992). The ancestral form is retained in elasmobranchs and sturgeons.

cichlid (teleost) fish optic nerve (Mack and Wolburg, 2006), and between mammalian olfactory (glial) ensheathing cells around growing axons (Mack and Wolburg, 1986; Miragall et al., 1994). These observations suggest that astrocytic glia in certain neural locations may be able to create diffusion barriers around critical local microenvironments. In pathologies where the mammalian brain endothelium is compromised (e.g., in tumors, epilepsy) (Bronger et al., 2005; Marroni et al., 2003), and in the glial scar around areas of damage, compensatory changes are observed in the glia, further revealing their barrier and regulatory potential (Abbott et al., 2006).

The new information from sturgeon, bichirs, and lungfish strongly supports the view that the glial barriers of elasmobranchs and sturgeon are archaic features, retained in these fish groups that occupy particular aquatic niches in which these features have not been too disadvantageous (Bone et al., 1995; Hickman et al., 2004). Elasmobranchs, sturgeons, and cephalopod mollusks all show some evidence of transition from a glial (ectodermal) to an endothelial (mesodermal) barrier, but with significant differences consistent with this being an independent evolutionary process in each group.

The BBB in sturgeon has some similarities with that of an invertebrate, the cuttlefish *Sepia officinalis* (Cephalopoda; Phylum Mollusca). *Sepia* has a low BBB permeability comparable to that of mammals (Abbott et al., 1985); in capillaries the endothelium is incomplete and the barrier is formed by a layer of perivascular glia, while in arterioles the endothelial/pericyte layer appears to form the barrier (Abbott and Bundgaard, 1992; Bundgaard and Abbott, 1992). In *Sepia* as in sturgeon, the glial barrier appears not to be the result of classical tight junctions (Lane et al., 1992), but rather to involve a greatly extended diffusional pathway formed by overlapping glial lamellae (Bundgaard and Abbott, 1992); further restriction may be provided by a "fibre matrix" plug within the extracellular cleft (Abbott et al., 1992; Lane and Abbott, 1992; cf. Curry and Michel, 1980). In sturgeons the extended zones of gap-junction-like contacts between the lamellae of the perivascular glial sheath may also facilitate the formation of a proteinaceous matrix plug as part of a glial barrier. The phenotypic differences in CNS barriers found among invertebrates, and among different fish lineages, are part of the evidence that such barriers evolved relatively independently several times in evolution.

The new evidence is best accommodated in cladogram Fig. 9B. In this scheme, the barrier located at the glial level in invertebrates continues into the ancestral vertebrate(s), then shifts to an endothelial location in the course of evolution, as a result of the significant selective advantage this gave. Advantages could include the division of labor between endothelium and glia. In tetrapods, the endothelium became specialized for regulating permeability and transport at the blood-brain interface, while glial cells became more involved with neuronal support and induction/communication within the gliovascular unit (Abbott, 2002; Abbott et al., 2006). Moreover, the glial syncytium separated from the blood-brain interface is then better able to act as an efficient spatial buffer for K^+, and means of water redistribution within the brain (Abbott, 2004; Dolman et al., 2005; Kimelberg, 2004).

Figure 9B implies that an endothelial BBB arose at least six times in evolution (convergent evolution), since all the crucial branch points with shift from glial (blue) to endothelial (red) are 400Myr or older (Kumar and Hedges, 1998) (see Fig. 1). The marked differences in the barrier between groups showing transitional BBB features, as well as between the endothelial barriers of different fish lineages (Agnatha, holocephalans, bichirs, teleosts, lungfish), is consistent with this idea, showing that there may be several different ways in which an

effective BBB can be achieved as a result of Darwinian evolution.

ACKNOWLEDGMENTS

The authors are grateful to Prof C. Nicholson and Prof R. Nieuwenhuys for comments on the MS, to Dr C.P. Hickman and Mr W. Ober and McGraw Hill for permission to reproduce fish illustrations.

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