The Blood–Brain Barrier, Amino Acids and Peptides

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Contents

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The suggestion for this review came from my dear friend, Professor Mortyn Jones, the neuroendocrinologist, who tragically died of cancer at the peak of his career. I would like to dedicate this review to him and I am sorry he is not with us to give us the benefit of his razor sharp mind. Since my main interest is amino acids, I have asked my colleague Berislav Zlokovic to join me in writing this review, which he has done with great skill, leaving me with less to write and more to edit than I expected.

We both wish to thank our great friends and mentors, Academician Professor Hugh Davson and Ljubisa Rakic, without whom our progress in science would have been limited. We also wish to thank Ms. Fiona Donaldson who typed the manuscript and tolerated the many changes we made.

Malcolm Segal
Berislav Zlokovic

1 Introduction

Definition of the barrier

The interstitial fluid (ISF) of the brain is separated from the blood by the blood–brain barrier (BBB). This barrier must not be thought of as a single entity or as an absolute restriction to all molecules, but as a multiple structure located at several sites within the brain. The first of these interfaces is located at the endothelium of the brain capillaries. Secondly there is a potential site for interchange on the outer linings of the brain between the dura and the arachnoid membranes. Thirdly, there are the choroid plexuses and the cerebrospinal fluid (CSF) which is in contact with the very permeable internal ependymal lining of the brain (Figure 1.1). Finally there are areas of the brain which lack a blood–brain barrier. These areas constitute the circumventricular organs and have leaky capillaries with a barrier at the ependyma which limits the spread of molecules from those regions (Figure 1.2). Each of these BBB sites has its own characteristic permeability and transport functions. We will now consider the properties of each of these barrier sites between the blood and the brain.

THE CEREBRAL CAPILLARY ENDOTHELIUM

Morphology

This interface has both the largest surface area and the shortest path length between the blood and the brain ISF. The cerebral capillaries, which at first sight seem little different from the rest of the systemic circulation, are in fact unique to the brain. Systemic capillaries are divided into two types (a) fenestrated, and (b) continuous, which refer to their observed morphological appearance.

The majority of the cerebral circulation is composed of continuous capillaries where the endothelium forms a complete double layer and contains a reasonable quantity of cytoplasm and intracellular organelles. These capillaries do, however, differ from other continuous capillaries in that the cytoplasm of the cerebral endothelial cells contains many more mitochondria

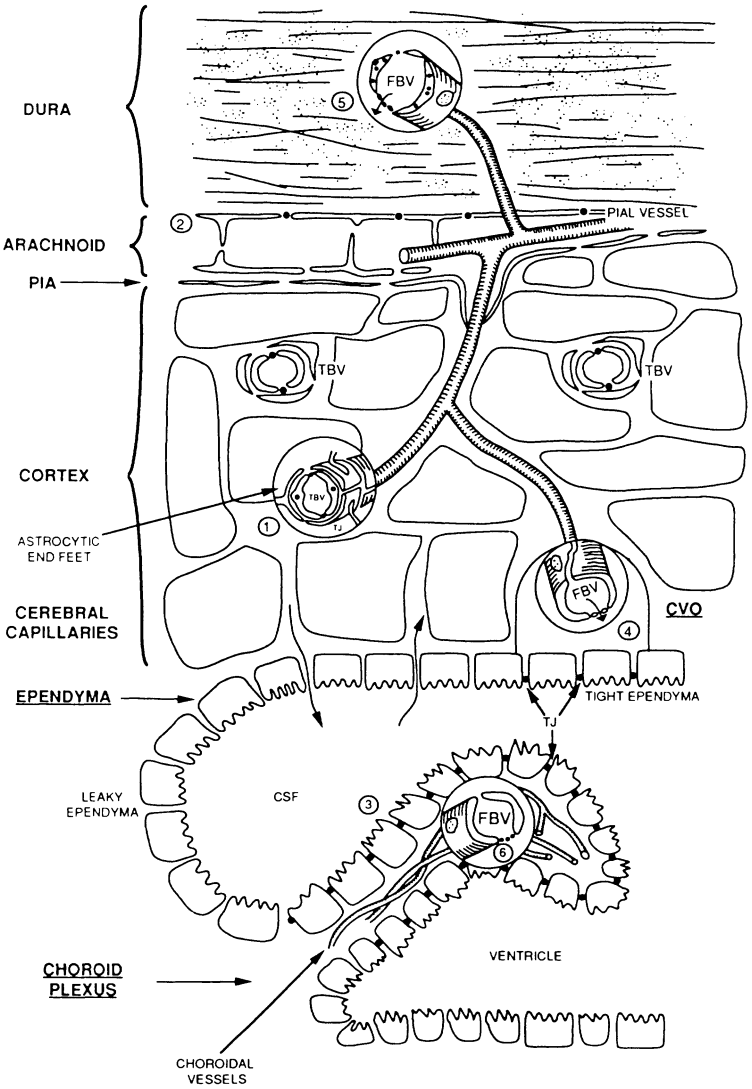

Figure 1.1 A diagram showing the three sites of the barrier between the blood and brain. (1) The cerebral capillaries (TBV) with tight junctions (TJ) sealing the endothelial cell margins. (2) The outer layer of the arachnoid also with TJ. (3) The epithelium of the choroid plexus joined by TJ except at the base. (4) Certain regions lack a barrier, the circumventricular organs (CVO) with fenestrated capillaries (FBV). For ependyma is leaky except in regions close to the CVOs where there are TJs. (5) Dural vessels are outside the barrier. (6) FBV are also found in the choroid plexus

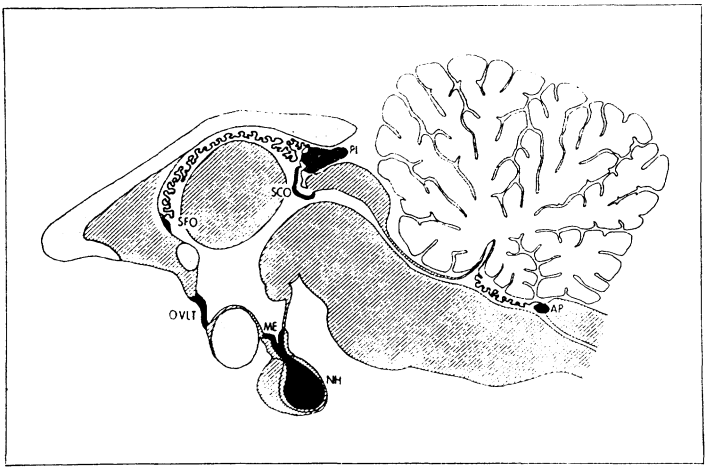

Figure 1.2 Section through squirrel monkey brain in median sagital plane. Most parts of venticular walls are covered by ciliated ependyma. Specialized non-ciliated ependymal cells cover circumventricular organs. These and similarly non-ciliated epithelium of choroid plexus of third and fourth ventricle are outlined in black. Located around third venticle are following circumventricular organs: median eminence (ME), neurohypophysis (NH), pineal body (PI), organum vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO), and subcommissural organ (SCO). Area postrema (AP) is found at transition of fourth ventricle into central canal. (From Weindl and Joynt, 1972[55] with permission)

than any other capillaries, which may indicate that these cells are capable of considerable metabolic work[1]. Another feature is that cerebral capillaries often appear to be running through the middle of the endothelial cell, so these vessels are formed from a continuous 'tube' of endothelium with no 'seams'[2]. Also, in other systemic capillaries there is a gap between endothelial cells which constitutes a low resistance pathway for all small molecules between blood and ISF. In contrast, in the brain these 'gaps' between the cells of capillary endothelium are obstructed by a continuous belt of occluding tight junctions which are sites of high transendothelial resistance[3,4]. Finally, the most unusual feature of cerebral capillaries is that they are virtually enveloped by a 'tube' composed of astrocytic end feet which are projections of specialized glia. Many of these glia also have processes in contact with the ependymal lining of the ventricles and so appear to be a connection between the blood, the cerebrospinal fluid (CSF) and the brain ISF. The function of these glia is still a matter of debate but at present no firm conclusions can be drawn. However, it is now known that the astrocytic end feet do not form a continuous layer around the cerebral capillaries and electron dense macromolecules in the CSF can pass into the subcapillary space, so they do not constitute an additional barrier to the diffusion of substances from the blood to the brain ISF[5]. The cerebral circulation only has fenestrated capillaries in the choroid plexuses and circumventricular organs, which are regions with

special functions and will be discussed later. The dural capillaries are also fenestrated, but these lie outside the blood–brain barrier[6]. Fenestrated capillaries have regions of endothelial wall where the cytoplasm is excluded and the two sides of the endothelial wall have become fused. In these areas there are circular patches, the fenestrae, where the wall is extremely thin or absent. These fenestrae have been proposed as leak pathways with a high hydraulic conductivity[7] but this is disputed by some[8,9].

Permeability characteristics of cerebral capillaries

Krogh, in his 1948 Croonian address[10], likened the permeability characteristics of the blood–brain barrier to those of the cell wall. Substances which have a high oil/water partition coefficient, for example anaesthetics, fat soluble molecules and respiratory gases, cross easily and rapidly gain access to the brain from the blood[11,12]. In contrast, polar molecules exhibit various degrees of restricted permeability depending on their properties and the selectivity offered by the capillary endothelium. The simplest property of a molecule is its size and shape and in the case of the BBB even quite small molecules, such as glycerol and urea, have a slow rate of permeation into the brain. Urea, for example, penetrates so slowly that it has been used as a hypertonic intravenous fluid to withdraw water from the brain if cerebral oedema occurs during neurosurgery. Considering the fact that urea will equilibrate fully across a red cell membrane, this illustrates the remarkable restriction offered by the BBB[13,14]. Molecules such as sucrose (with a molecular weight of 300) are virtually excluded by this barrier[15]. In contrast, molecules such as D-glucose and various L-amino acids easily gain access to the brain, whereas others such as mannitol, L-glucose and glycine do not[16]. Since these permeating molecules demonstrate saturation, stereospecificity and competition, there can be little doubt that this permeation is a carrier mediated process[17]. Electrolytes are similarly obstructed by the BBB, which further demonstrates that size and charge play a crucial part in the restrictive nature of the BBB. Sodium, for example, will equilibrate with muscle within minutes yet takes 24–48 hours to fully equilibrate with the brain[18].

Routes of permeation through the cerebral endothelium

There are three possible routes through the wall of the brain capillary. Firstly molecules may pass directly through the wall and, as we discussed in the last section, this route depends primarily on the nature of the cell wall and its selectivity. Secondly, molecules may pass through intercellular clefts between endothelial cells. Studies with electron dense molecules, such as horseradish peroxidase, colloidal lanthanum, and the smallest of these molecules, microperoxidase (1800 Daltons), show that they all fail to penetrate the

intercellular clefts between endothelial cells of the cerebral capillary wall[3,4]. These studies have shown that the point of obstruction is the occluding band of tight junctions, the zonula occludens, which joins the endothelial cells together.

Freeze fracture studies, using scanning electron microscopy, have resolved the nature of these junctions into a series of strands; apparent correlation was found between the number of strands within the junctional complex and the resistance offered by the cell sheet[19–21]. However, subsequent studies have challenged this simple concept and tortuous open pathways have been found between the strands of some zonula occludens using serial sectioning techniques[21]. These morphological studies are limited by the molecules which can be visualized under the electron microscope and can only place a lower estimate on the size of this potential water-filled pathway as being less than 1.5 nm. However, since sugars, amino acids and electrolytes are much smaller than this and exhibit a limited permeability, the dimensions of the pathway for free diffusion across the blood–brain barrier must be less than 0.5 nm[22].

As has been stated, the permeability of the cerebral capillaries is many orders smaller than that of other systemic beds. Michel and his co-workers have shown that, although there is a potential open pathway between the endothelial cells of most capillary beds, this route is in fact filled with a fibrillar matrix of basement membrane. This matrix is charged and can restrain macromolecules such as albumin, even though the dimensions of the morphological path appear much greater[23,24]. In the brain the nature of this fibrillar matrix has not been considered and studies have concentrated primarily on the tight junctions. In passing, it is interesting to note that cerebral capillaries offer a considerable resistance to the movement of small molecules, so that the Starling equilibrium cannot apply to the brain. Having capillaries of such high resistance does permit these structures to limit the species of molecule which can cross the cerebral endothelium and it is this obstruction that removes the need for a lymphatic drainage system within the brain.

The third potential pathway is by the vesicles, seen within the cytoplasm and occasionally fusing the endothelial wall. The problem has been to resolve the dynamics of these structures and whether there is a net direction of movement. There can be little doubt that substances on either side of the cell can gain access to vesicles within the cytoplasm, but no one has as yet demonstrated a net direction of movement of these vesicles within the cytoplasm. The difficulty with these morphological studies is related to the slow nature of the fixation process and the volume of sections which need to be cut to serially section large areas of the capillary wall. Some prodigious studies have reconstructed the interaction of the vesicles with the cell wall and have demonstrated a potential path for macromolecules[25,26]. These studies have demonstrated chains of vesicles forming a continuous pathway across the cell, but these findings are vigorously challenged by other workers

in the field[27,28]. Recent studies with ultrathin sections following gold colloid may resolve this dispute[29]. However, if it is assumed that vesicles may contribute to macromolecular passage across the blood–brain barrier, it must not be assumed that this route is a non-specific bulk pathway, but one which appears to require that the macromolecule interacts with a specific membrane-bound receptor and the receptor molecule complex is then internalized. Experimental evidence, which will be discussed in Chapter 8, has demonstrated a receptor mediated uptake for some specific macromolecules such as the cationized form of albumin as well as a time dependent uptake of IgG. These findings now need to be correlated with morphological observations to determine the route that these molecules have taken in their passage from blood to the brain ISF.

Future studies must combine physiological, immunological and morphological studies to resolve the mechanism by which macromolecules can cross the physical restriction offered by the BBB and enter the brain. As will be discussed later, the physiologists' view of a capillary wall acting solely as a fixed cellular tube is now no longer tenable and the dynamic nature of endothelia, long familiar to the immunologists, must now be integrated into the functions of the blood–brain barrier.

Apparent barrier mechanisms – enzymes

A variety of neurotransmitters and peptides have one set of actions in the blood and a completely different role in the brain. For example, intravenous norepinephrine in the chicken causes excitement whereas if injected into the CSF it causes sleep[30]. This type of dual function requires that these agents in the blood be excluded from the brain. At first sight it seemed that this was achieved by the simple physical restriction of the BBB. However, studies with labelled molecules have shown that the label was able to penetrate from blood to brain. More careful studies have revealed that although minute trace amounts do cross the BBB, the bulk of the compound is degraded either by enzymes in the blood or by specific enzymes bound to the wall of the cerebral capillaries[31,32].

This type of study can easily lead to misinterpretation, since when molecules such as peptides are degraded to their constituent amino acids which can be labelled and are easily transported, they will give the appearance of an uptake of the peptide[33]. It is thus always essential to check the integrity of the macromolecule by a specific identification process, before the uptake of these molecules can be substantiated (see Chapter 8).

THE DURA, ARACHNOID AND PIA

The external coverings of the brain, the meninges, are composed of the dura, the arachnoid and the pia and form a potential interface with the external surface of the brain and the spinal cord. The dura contains fenestrated capillaries so that large molecules can pass from the blood into the dura. However, further passage towards the brain is limited by the external layer of the arachnoid, the cells of which are joined together by tight junction[34]. The blood vessels within the pia are all of the continuous type seen elsewhere in the brain so constituting the BBB in these regions. However, studies on isolated pieces of the arachnoid membrane and with *in vivo* perfusions of the ventricle to the subarachnoid space do point to this layer as having some transport capabilities[35,36]. There is, however, a problem in ensuring that the needles used to perfuse or to collect from the subarachnoid space are actually beneath the outer arachnoid membrane and not in the subdural space. The same problem may be encountered with cups on the brain surface which have been used to study uptake by the brain *in vivo*[37]. If there is an intact outer arachnoid membrane in these methods there will be little movement towards the brain since this layer is the site of the BBB at this interface. However, a large clearance of substances may still be observed, since if the fluid is in contact with the dural vessels, which are outside the BBB, molecules will pass easily into the bloodstream as these vessels are of the permeable systemic type[38].

CHOROID PLEXUSES AND CSF

The ventricles and subarachnoid space of the brain and spinal cord are filled with cerebrospinal fluid (CSF). This fluid, which is formed by the choroid plexuses within the ventricles, slowly flows through these cavities into the subarachnoid spaces where it drains back into the blood via the arachnoid granulations, the spinal nerve roots and the olfactory tracts[39–41].

The choroid plexuses are well vascularized organs; those in the lateral and third ventricles are double-sided leaf-like structures, whereas that in the fourth ventricle is a single-sided structure dividing this ventricle from the cisterna magna. The capillaries of the choroid plexuses are of the 'leaky' fenestrated type, but although macromolecules can gain access to the interstitial space of the plexuses, further progress towards the CSF is halted by an occluding band of tight junctions joining the cells of the choroidal epithelia together close to the CSF side of the tissue[42–44]. The choroid plexuses are less of a barrier than that found within the capillaries of the brain, so molecules can pass slightly more easily from blood to CSF than from blood to brain ISF[45,46]; however, the area of interface offered by the choroidal interface is 1/5000 of that of the cerebral capillaries. These structures, as well as secreting CSF, can secrete proteins and are capable of transporting

electrolytes, sugars, amino acids and other molecules between blood and CSF, so forming an important element of the brain homeostatic mechanism[47–49].

It is of interest that the cells which cover the choroid plexuses are continuous with the ependymal linings of the ventricles. Many studies have shown that the ependyma is an extremely leaky membrane so that substances in the CSF freely exchange with the brain ISF[50].

At the base of the choroid plexuses, there is a sudden transition between 'tight' choroidal epithelia and the leaky ependyma. Since the choroidal blood vessels are fenestrated, there is a potential leak pathway which may account for the entry of lanthanum from blood into CSF by this route[51,52]. However, since the concentration of macromolecules is low in CSF, this route must be limited primarily by the long path length and slow diffusion, so in fact it makes little real contribution to the exchanges between blood and CSF.

SPECIAL REGIONS OF THE BRAIN. THE CIRCUMVENTRICULAR ORGANS

Whilst the vast majority of the capillaries of the brain are of the continuous 'tight' type, a few sites do exist, where the capillaries are 'leaky' and the barrier absent. These sites constitute a group of special brain regions, the circumventricular organs[53], so called since they are all located close to the ventricles of the brain. These regions were originally identified by dye studies and staining was seen in the area postrema, median eminence, neuro hypophysis, pineal gland, the organum vasculosum of the lamia terminalis and the subcommisural organ as well as the choroid plexuses. These structures are now known to be involved in the salt balance, hormonal feedback and release processes and are under intense investigation (see Chapter 4). It is thus obvious that free exchange is necessary between blood and brain for their functions, but these sites do not constitute a route of free access to other regions of the brain, since the area is bounded by a region of ependyma with tight junctions, so limiting escape of molecules from these organs[54].

CONCLUSION

The blood–brain barrier is a multiple structure situated at the cerebral capillary endothelium, the choroid plexuses and the outer arachnoid membrane. Each of these sites can act as a simple physical barrier, restraining the movement of molecules and ions, the degree of restraint being largely governed by lipid solubility.

In addition to this simple physical barrier, these interfaces contain a variety of transport proteins, which can move electrolytes, sugars, amino acids and other non-electrolytes between the blood and brain and CSF, often

against an existing concentration gradient. Enzymes in blood and those bound to the cerebral endothelium can denature molecules attempting to cross the BBB.

Finally, these structures may also transport intact macromolecules by specific processes, the mechanism of which is at present poorly understood.

REFERENCES

1. Oldendorf, W.H. and Brown, W.J. (1975). Greater number of capillary and endothelial cell mitochondria in brain than in muscle. *Proc. Soc. Exp. Biol. Med.*, **149**, 736–738
2. Oldendorf, W.H., Cornford, M.E. and Brown, W.J. (1977). The large apparent work capability of the blood brain barrier: a study of the mitochondrial content of capillary endothelial cells in brain and other tissues. *Ann. Neurol.*, **1**, 409–417
3. Reese, T.S. and Karnovsky, M.J. (1967). Fine structural localization of a blood–brain barrier to exogeneous peroxidase. *J. Cell Biol.*, **34**, 207–217
4. Feder, N. (1971). Microperoxidase an ultrastructural tracer of low molecular weight. *J. Cell Biol.*, **51**, 339–343
5. Brightman, M.W. (1967). The intracerebral movement of proteins injected into blood and cerebrospinal fluid of mice. *Prog. Brain Res.*, **29**, 19–37
6. Andres, K.H. (1967). Uber die Fienstanktur der Arachnordea und Duramater von Mammalie. *Z. Zellforsch.*, **79**, 272–295
7. Simionescu, N., Simionescu, M. and Palade, G.E. (1972). Permeability of intestinal capillaries pathway followed by dextrans and glycogens. *J. Cell Biol.*, **53**, 365–392
8. Renkin, E.M. (1978). Transport pathways through capillary endothelium. *Microvasc. Res.*, **15**, 123–135
9. Artuson, G. and Granath, K. (1972). Dextrans as test molecules in studies of functional ultrastructure of biological membranes. *Clin. Chim. Acta*, **37**, 309–322
10. Krogh, A. (1946). The active and passive exchanges of inorganic ions through the surfaces of living cells and through living membrane generally. *Proc. R. Soc. Biol.*, **133**, 140–200
11. Rappoport, S.I., Ohno, K. and Pettigrew, K.O. (1979). Drug entry into the brain. *Brain Res.*, **172**, 354–359
12. Cornford, E.M., Braun, L.D. and Oldendorf, W.H. (1982). Comparison of lipid mediated blood–brain permeability in neonates and adults. *Am. J. Physiol.*, **243**, C161–168
13. Kleeman, C.R., Davson, H. and Levin, E. (1962). Urea transport in the central nervous system. *Am. J. Physiol.*, **203**, 739–747
14. Crone. C. (1965). The permeability of brain capillaries to non-electrolytes. *Acta Physiol. Scand.*, **64**, 407–417
15. Davson, H. and Bradbury, M.W.B. (1965). The extracellular space of the brain. In: *Biology of Neuroglia*, eds., E.D.P. De Robertis and R. Carrea. (Elsevier, Amsterdam)
16. Oldendorf, W.H. (1971). The brain uptake of radiolabeled amino acids, amines and hexoses after intra arterial injection. *Am. J. Physiol.*, **221**, 1629–1639
17. Oldendorf, W.H. and Szabo, J. (1976). Amino acid assignment to one of three blood–brain barrier amino acid carriers. *Am. J. Physiol.*, **230**, 94–98
18. Davson, H. and Pollay, M. (1963). The turnover of ^{24}Na in the cerebrospinal fluid and its bearing on the blood–brain barrier. *J. Physiol.*, **167**, 247–255
19. Claude, P. and Goodenaugh, D.A. (1973). Fracture faces of zonula occludens from tight and leaky epithelia. *J. Cell Biol.*, **58**, 390–400
20. Connell, C.J. and Mercer, K.L. (1974). Freeze-fracture appearance of the capillary endothelium in the cerebral cortex of mouse brain. *Am. J. Anat.*, **140**, 595–598

21. Van Deurs, B. and Koehler, J.K. (1979). Tight junctions in the choroid plexus epithelium. *J. Cell Biol.*, **80**, 662–673
22. Bradbury, M.W.B. (1979). *The Concept of the Blood–Brain Barrier*. (Wiley, Chichester)
23. Michell, C.C. (1985). The Malpighi Lecture. Vascular permeability – the consequence of Malpighi's hypothesis. *Int. J. Microcirc. Clin. Exp.*, **4**, 265–284
24. Curry, F.E. and Michel, C.C. (1980). A fiber matrix model of capillary permeability. *Membrane Res.*, **20**, 96–99
25. Simionescu, N., Simionescu, M. and Palade, G.E. (1975). Permeability of muscle capillaries to small heme-peptides. Evidence for the existence of patent trans-endothelial channels. *J. Cell Biol.*, **64**, 586–607
26. Renkin, E.M. (1979). Multiple pathways of capillary permeability. *Circ. Res.*, **41**, 735–743
27. Bundgaard, M. (1980). Transport pathways in capillaries: in search of pores. *Ann. Rev. Physiol.*, **42**, 325–336
28. Frøkjaer-Jenson, J. (1983). The plasma luminal vesicular system in capillary endothelium conventional electron microscope (EM) thin sections compared with the picture arising from ultrathin (~140Å) serial sectionary. *Prog. Appl. Microcirc.*, **1**, 17–34
29. Müllgard, K., Balslev, Y. and Saunders, N.R. (1988). Structural aspects of the blood–brain and blood–CSF barriers with regard to endogenous proteins. In: *Peptide and Amino Acid Transport Mechanisms in the Central Nervous System*, eds., Lj. Rakic, D.J. Begley, H. Davson and B.V. Zlokovic, (Macmillan, London), pp. 93–102
30. Dewhurst, W.G. and Marley, E. (1965). Action of sympathomimetic and allied amines on the central nervous system of the chicken. *Br. J. Pharmacol.*, **25**, 671–727
31. Draskoci, M., Feldbury, W. and Haranath, P. (1968). Passage of circulating adrenalin into perfused cerebral ventricles and subarachnoid space. *J. Physiol.*, **150**, 24–49
32. Björklund, A., Falck, B., Hromek, F. and Owman, Ch. (1969). An enzyme barrier mechanism for monoamine precursors in newly-forming brain capillaries following electrolytic or mechanical lesions. *J. Neurochem.*, **16**, 1605–1608
33. Zlokovic, B.V., Begley, D.J., Segal, M.B., Davson, H., Rakic, Lj., Lipovac, M.N., Mitrovic, D.M. and Jankov, R.M. (1988). Neuropeptide transport mechanisms in the central nervous system. In: *Peptides and Amino Acid Mechanisms in the Central Nervous System*, eds., Lj. Rakic, D.J. Begley, H. Davson and B.V. Zlokovic, (Macmillan, London), pp. 3–20
34. Nabeshima, S., Rose, T.S., Landis, D.M.O. and Brightman, M.W. (1975). Junctions in the meninges and marginal glia. *J. Comp. Neurol.*, **164**, 127–170
35. Lorenzo, A.V. and Snodgrass, S.R. (1972). Leucine transport from ventricles and the cranial subarachnoid space in the cat. *J. Neurochem.*, **19**, 1287–1298
36. Wright, E.M. (1974). Active transport of glycine across the frog arachnoid membrane. *Brain Res.*, **76**, 354–358
37. Levin, E., Sepulveda, F.V. and Yudilevich, D.L. (1974). Pial vessel transport of substances from cerebrospinal fluid to blood. *Nature (London)*, **249**, 266–267
38. McComb, J.G., Davson, H. and Hollingsworth, J.R. (1975). Further studies on the difference between ventricular and subarachnoid perfusion. *Brain Res.*, **89**, 81–91
39. Kido, D.K., Gomez, D.G., Pavese, A.M. and Potts, D.G. (1976). Human spinal arachnoid granulations. *Neuroradiology*, **11**, 221–228
40. Jackson, R.T., Tigges, J. and Arnold, U. (1979). Subarachnoid space of the CNS and nasal mucosa and lymphatic system. *Arch. Otolaryngol.*, **150**, 180–184
41. Bradbury, M.W.B., Cserr, H.F. and Westrop, R.J. (1981). Drainage of cerebral interstitial fluid into deep cervical lymph of the rabbit. *Am. J. Physiol.*, **240**, F329–336
42. Brightman, M.W.B. and Reese, T.S. (1969). Junctions between intimately apposed cell membranes in the vertebrate brain. *J. Cell Biol.*, **40**, 648–677
43. Segal, M.B. and Burgess, A.M.C. (1974). A combined morphological study of the secretory processes in the rabbit choroid plexus. *J. Cell Sci.*, **14**, 339–350
44. Van Deurs, B.G. and Koehler, J.K. (1979). Tight junctions of the choroid plexus epithelia. *J. Cell Biol.*, **80**, 662–673

45. Brightman, M.W., Prescott, L. and Reese, T.S. (1975). Intercellular junctions of special ependyma. In: *Brain Endocrine Interaction*. II. *The Ventricular System*. (Karger, Basel), pp. 146–165
46. Welch, K. and Sadler, K. (1966). Permeability of the choroid plexus of the rabbit to several solutes. *Am. J. Physiol.*, **210**, 652–660
47. Deane, R. and Segal, M.B. (1985). The transport of sugars across the choroid plexus of the sheep. *J. Physiol.*, **362**, 245–260
48. Preston, J.E., Segal, M.B., Walley, G. and Zlokovic, B.V. (1989). The uptake of amino acids by the choroid plexus of the sheep. *J. Physiol.*, **408**, 31–43
49. Martinez-Palomo, A. and Erlig, D. (1975). Structure of tight junctions in epithelia with different permeability. *Proc. Natl. Acad. Sci. USA*, **72**, 4487–4491
50. Brightman, M.W. (1965). The distribution within the brain of ferritin injected into the cerebrospinal fluid compartments. *J. Cell Biol.*, **26**, 99–123
51. Baulden, T.W. and Krigman, H.R. (1975). Differential permeability of cerebral capillary and choroid plexus to lanthanum ion. *Brain Res.*, **99**, 444–448
52. Van Deurs, B., Moller, M. and Amtorp, O. (1978). Uptake of horseradish peroxidase for CSF into choroid plexus of the rat with special reference to transepithelial transport. *Cell Tissue Res.*, **187**, 215–234
53. Davson, H., Welch, K. and Segal, M.B. (1987). *The Physiology and Pathology of the Cerebrospinal Fluid*. (Churchill Livingstone, Edinburgh)
54. Reese, T.S. and Brightman, M.W. (1968). Similarity of structure of epithelia overlying fenestrated cerebral capillaries. *Anat. Res.*, **160**, 414
55. Weindl, A. and Joynt, R.J. (1972). Ultrastructure of the ventricular walls. *Arch Neurol.*, **26**, 420–427

2 Methods for the study of the functions of the blood–brain barrier

The blood–brain barrier is a complex multiple site structure with both active and passive elements. Any methods used to study such a system will always encounter a number of limitations and inbuilt errors. The recent developments in pharmacology, immunology and molecular biology have provided a variety of new techniques, which, in conjunction with the need for a greater understanding of brain pathology, have given a fresh impetus to the development of new methods to study the blood–brain barrier. However, it is essential that in the interpretation of data obtained from these new methods both the underlying limitations of the method and the complexity of the blood–brain barrier be always kept in mind. This is especially critical when such results are used to extrapolate from the *in vitro* experiment to the whole animal or the extrapolation of animal data to man. In addition, kinetic constants and parameters, which can be used to quantify barrier function and which have been derived by different experimental methods, should always be subject to the appropriate mathematical transformation so that the data can be compared between various methods and species.

The methods used to study the functions of the blood–brain barrier can be divided broadly into two main groups depending on whether the method uses the whole animal, i.e. *in vivo*, or *in vitro* where the barrier is simplified in some way. In this latter group, the most widely used *in vitro* preparation has been that of isolated cerebral capillaries harvested from homogenized brain. An alternative approach has been to grow these endothelial cells in tissue culture on a supporting mesh to form a pure 'barrier' *in vitro*. Even greater simplification has been achieved by fragmentating the endothelial cells of these capillaries and studying the characteristics of potential transport proteins in these cell wall fragments.

The *in vivo* studies may be further classified. Firstly, the main subdivision depends on the particular interface of the blood–brain barrier under study and on the direction of movement, e.g. blood to brain, blood to CSF, CSF to

blood, etc. The molecule under study can be placed in the compartment under consideration and the interaction of the molecule studied either at the cellular interface of the barrier or on the movement across the barrier between compartments.

IN VIVO TECHNIQUES

Blood to brain techniques

The methods used to study the movement of molecules from blood to brain can be divided on the basis of the length of time during which the interaction has been studied, i.e. rapid, intermediate or long-term. The choice of method for a given molecule is highly dependent on its stability in blood and on the rate of its interaction with barrier, which emphasizes the need for a complementary approach, rather than the limitations of the different methods. Among the most frequently used rapid kinetic studies are the intracarotid injection technique of Oldendorf[1] and the paired-tracer indicator dilution method developed originally by Chinard for the lung[2] and applied to brain by Crone[3] and by Yudilevich[4]. For molecules which have a slow uptake and cannot be detected in the brain during single capillary passage through the cerebral circulation, different approaches have been developed. Most of these are based on the Davson's[5] classical steady-state kinetic analysis of solute entry into the brain, but deviate from it in certain ways, particularly in the calculation of permeability surface area product (PS) and the constants of saturable processes, K_m and V_{max}, which can be derived from the PS-value. From the technical view point these long-term kinetic studies can be further classified on the basis of i.v. injection with single-brain sampling or multiple-brain sampling analysis, such as, for example, the method of Ohno *et al.*[6] and the experimental approaches of Gjedde[7], Patlak *et al.*[8], Blasberg *et al.*[9] and Baños *et al.*[10]. In contrast to these methods is the vascular brain perfusion technique of Zlokovic *et al.*[11], which can be used to keep the concentration constant in the perfusate of a slowly-penetrating compound for a sufficient length of time (up to 20 min) to characterize and quantitate the interaction of these molecules at the blood–brain barrier. The vascular brain perfusion method has also been applied to characterize the transfer of rapidly penetrating molecules (up to 1 min) using the technique of Takasato *et al.*[12].

The final most exciting method for the study of the entry characteristics from blood to brain is Syrota's use of positron emission tomography in man[13]. This relatively non-invasive method will yield much data of interest both from the clinical and experimental points of view.

Blood/CSF methods

The most frequently used 'CSF-to-blood' technique without doubt is that of ventriculo-cisternal perfusion; this has been used in animals as diverse as the dogfish, rat and even man. Other methods include intracerebroventricular injection techniques and perfusion from the ventricles to the aqueduct of Sylvius. These 'blood to CSF' methods mostly use the classical steady-state blood level approach, with sampling of CSF from the ventricles, cisterna magna or lumbar sac and the calculation of CSF/plasma ratios. A more specific approach has involved sampling the nascent CSF from the surface of the oil covered choroid plexus *in vivo*, which was developed by Ames *et al.*[14] and by Welch *et al.*[15]. Miner and Reed[16] used an alternative method by enclosing the plexus in a capsule *in vivo*. The choroid plexus may be isolated further by the use of the *in vitro* isolated, blood perfused, choroid plexus of the sheep, a technique developed by Pollay *et al.*[17] and used extensively by Segal[18].

In the present volume it is not possible, due to space limitations, to provide a detailed and comprehensive description of all the above mentioned methods, and we have decided to focus our interest primarily on techniques employed in peptide and amino acid transport studies at the blood–brain barrier.

IN VITRO TECHNIQUES

Isolated cerebral microvessels

For more than 15 years the blood–brain barrier has been studied *in vitro* by means of isolated brain microvessels. Joó and Karnushina[19] developed the first micromethod for isolating cerebral microvessels, and since then a number of similar techniques have been used to separate the vessels from the rest of the brain tissue. Briefly, the isolation procedure can be summarized in two steps: (1) homogenization of the brain and/or gray matter with a loosely fitting rotating pestle (collagenase digestion may also be applied) and (2) separation of the vessels by either sieving through nylon nets and/or density gradient centrifugation, or passage through a column of glass beads. Alternatively during the separation phase these procedures may be combined.

The need to study the blood–brain barrier physiology and pathology *in vitro* arose from the fact that the barrier is difficult to study in isolation *in vivo* since it is located at the continuous endothelium of cerebral blood vessels. This special endothelium functions with respect to its permeability and electrophysiological characteristics, like a transporting epithelium[20]. However, experimental models used to study the physiology of epithelial tissues *in vitro*, such as the Ussing chamber for the frog skin, or isolated renal microperfused tubules, offer a much better possibility for transport research

compared to isolated cerebral microvessels. In the above epithelia, continuity of the cellular layer remains preserved during the isolation procedure which allows the cellular barrier to be studied as a membrane separating two compartments, so that the net flux of ions and the electrical parameters of the epithelium may be investigated.

In spite of its limitations, the isolated microvessel preparation has proved particularly useful in the study of the biochemical properties of the cerebral vasculature, such as the lipid composition of the endothelial cells, the amino acid and carbohydrate composition of the basement membrane and the determination of major protein components[21]. In addition, a number of different enzymatic activities[22] and the presence of various classical neurotransmitters and enzymes involved in their metabolism[23] have been shown. Other enzymes involved in the metabolism of cyclic nucleotides, including adenylate cyclase, guanylate cyclase and cyclic phosphodiesterase[24], have also been demonstrated. The preparation has been of particular interest in monitoring receptor-mediated transcytotic exchanges of larger molecules[25] as well as being successfully applied in various receptor-binding studies[26]. This technique has proved to be a powerful tool for studying the molecular pathology of brain microvessels, including responses to cholesterol feeding and hypertension, effects of ischaemia and brain oedema, effects of lead intoxication[21,27] and, more recently, characterization of amyloid angiopathy in Alzheimer's disease[28].

Although this preparation is most useful for above-mentioned purposes, it has been suggested that it can be inappropriate or even misleading for others[29]. For example, most studies of transport with the preparation have examined the uptake of radiolabelled solutes into the suspended tissue fragments. These disrupted sections of microvessels are usually about 100μm in length and may be open at their broken ends so that both luminal and abluminal sides can contribute to the measured cellular uptake or binding[29]. However, in some cases, as for example for Na/K-ATP-ase, the exact localization to the abluminal side of the endothelium has been demonstrated[30]. It has been computed on the basis of surface area–volume relationships that rapidly penetrating molecules, such as glucose and some amino acids, may reach a half-steady-state equilibrium very rapidly, within 5–10 s[31]. The estimated kinetic constants may be very different from those obtained *in vivo*, as well as varying between values for isolated microvessels prepared by different groups. Some technical difficulties reside in the difficulty of obtaining a relatively pure preparation of the capillaries. The isolated cerebral capillaries may be contaminated with small arterioles and venules, with their smooth muscle, as well as substantial astrocytic contamination, and this may be responsible for a relatively large variation in computed kinetic constants between different groups.