

Dynamic *in vitro* modeling of the blood–brain barrier: a novel tool for studies of drug delivery to the brain

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Apart from in exceptional and rare circumstances when drugs may be inserted directly into the CNS, it is often difficult to achieve therapeutically relevant dosages in the brain. This is because oral or parenteral administration effectively results in the exclusion of most drugs by the blood–brain barrier (BBB). Therefore, it is important to develop systems that allow for rapid and inexpensive determination of the BBB-permeability properties of novel therapeutic drugs. Recent advances allow for the manipulation of brain transport of drugs by chemical facilitation with lipophilic compounds or viral vectors. Studies performed on viable *in vitro* models are set to accelerate the design of drugs that selectively and aggressively target the CNS.

The method by which a relatively undifferentiated and morphologically simple EC can specialize within such a complex physiological machinery remains largely unknown, but both parenchymal and intravascular factors appear to be involved. Perhaps paradoxically, *in vitro* experiments have provided more information on the brain's microvascular EC physiology than observations performed *in situ*^{3–5}. The latter are almost impossible with the techniques that are currently available. Early attempts to grow brain ECs *in vitro* have not, however, been devoid of challenges, and it soon became apparent that most of the typical properties of BBB endothelium reluctantly survive the steps necessary for the isolation and culturing of vascular ECs⁴. Partial success was achieved upon the discovery that expression of some of the salient properties of ECs (such as tight junctions) was greatly enhanced by exposure to glial factors, or glia themselves^{6,7}. Other phenotypic aspects of the *in situ* BBB were more difficult to reproduce, and low trans-endothelial electrical resistance (TEER) values and abnormally high permeability characterized both 'monoculture' and 'co-culture' models^{5,8–10}. Surprisingly, it was not until recently that emphasis has been placed on the possibility that both flow-dependent phenotypic expression and glial influences may be important conditions of *in vitro* modeling of the BBB¹¹.

Advances in molecular neurobiology are improving understanding of the etiology of chronic and acute degenerative brain disorders. A predictable result of the increased knowledge and awareness of CNS dysfunction mechanisms is the development of pharmaceutical strategies to treat a broad spectrum of neurological disorders, such as stroke, brain tumors, substance abuse and dementia. An equally foreseeable scenario resulting

▼ The mammalian blood–brain barrier (BBB) is comprised of microvascular endothelial cells (ECs). These cells acquire specialized properties upon exposure to as yet unknown factors present in the CNS, and are presumably secreted by neighboring glia^{1,2}. Among the crucial properties of BBB endothelia is the capacity to act as a virtually impenetrable barrier, thus isolating the brain from systemic influences, while simultaneously providing a pathway for the transport of nourishment to neurons buried in the brain parenchyma. In addition, the process of clearance of potentially toxic substances from the brain into the blood also relies on BBB-related mechanisms. The vital nature of this function becomes apparent if one considers the volumetric rigidity of the skull. This, together with the lack of lymphatic drainage from the CNS, implies that even a small accumulation of proteins and water into the CNS requires immediate and complete clearance by mechanisms other than lymph circulation.

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from the rapid pace of modern neuroscience is that most of the promising therapeutic approaches will fail the clinical challenge. This is because most of the drugs that act on the CNS are effectively excluded by the BBB. Both drug design and strategic planning of CNS chemotherapy are based on current understanding of the intrinsic exclusion properties of cerebral ECs. In the last few years however, it has been discovered that drug delivery across the BBB is not only hindered by the lipophilic nature of the plasma membrane, but also by numerous and as yet not fully understood active processes of drug exclusion. This is particularly true for substances such as antitumorals, which are appropriately perceived as noxious by the endothelium and as such are excluded from the delicate intricacies of the CNS.

At least two approaches have been used in attempts to overcome this predicament: the development of strategies aimed at the opening of the BBB and the design of substances suitable for targeted CNS delivery. Because of the extremely large number of compounds to screen and the highly unpredictable fate of drugs at the blood–brain interface, it is hardly surprising that several attempts have been made to develop a reliable and ‘prophetic’ cell culture-based (or theoretical) model of the BBB.

In vitro modeling of the BBB

Traditional bidimensional models

Because of the topographical obstacles associated with the direct investigation of brain microvessels *in vivo*, the structure and physiological properties of the BBB have been extensively studied in isolated microvessels and in primary cultures of brain microvascular ECs. Based on the authors’ understanding of ECs *in situ*, an *in vitro* model must reproduce all of the following properties:

- expression of tight junctions between ECs and the relative lack of pinocytotic vesicles. This is commonly assessed by measuring TEER or permeability to radioactive molecules of poor or negligible permeation (such as sucrose, mannitol);
- selective (and asymmetric) permeability to physiologically relevant ions, such as Na⁺ and K⁺; expression of site-specific pumps and ion carriers;
- selective permeability to molecules, based on their molecular weight and oil/water partition coefficient;
- expression of BBB-specific transporters for metabolic substrates or building blocks necessary for neuronal and glial cell physiology;
- functional expression of mechanisms of active extrusion of otherwise permeable substances (such as antineoplastic agents).

In addition, it is sometimes useful to immunohistochemically localize BBB markers in ECs; the presence or absence of these markers sometimes correlates poorly with the predictive

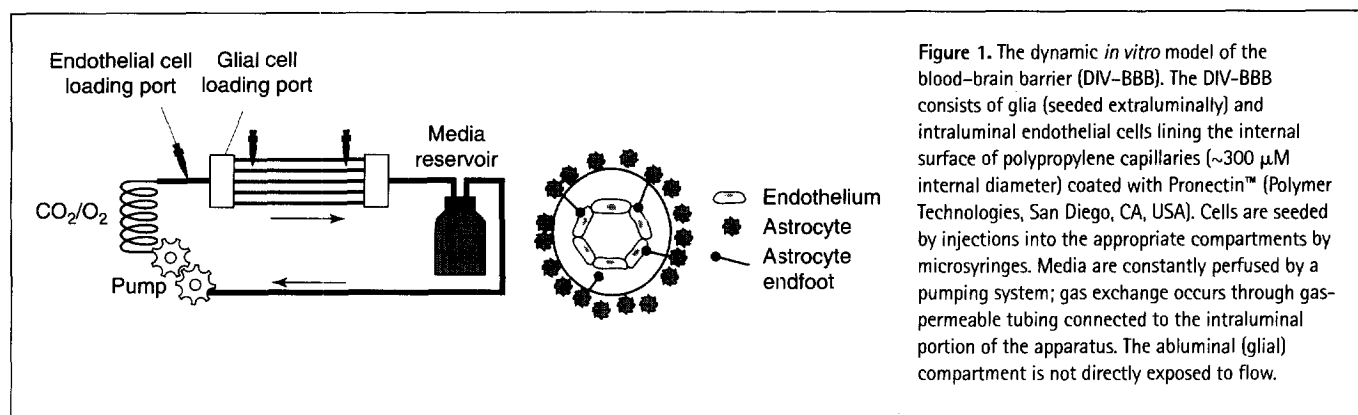
properties of a given model, and the expression of a particular set of markers in a model characterized by abnormal paracellular leak does not constitute a valid experimental paradigm for drug delivery studies.

Bidimensional co-culture models have been created through the use of modified petri dish devices. These consist of a plastic support that holds a transparent and porous material in which endothelial cells are seeded. Typically, astrocytes or other glial cells are grown on the opposing surface to mimic the topographic separation of endothelial and glial cells *in situ*. These systems allow extensive testing of relatively large numbers of compounds, but are usually distinguished by nonphysiological trans-endothelial ‘leak’, thus making it difficult to predict exactly permeation across the *in situ* BBB. In addition, even ECs of CNS origin tend to lose their exclusive properties rapidly upon *in vitro* culturing.

In an attempt to improve the TEER (and therefore to decrease non-specific leakage) towards more physiological values, several investigators have engineered brain-like ECs. This has been achieved through the use of molecular techniques aimed at introducing into non-BBB cells genes that are involved in the regulation of BBB-specific markers^{12–15}. While these sophisticated manipulations have improved the resistance across these cultured endothelia, several specific markers of BBB function undergo altered expression or are lost during the process. Since the recognition of the contribution of glia (or glial factors) to BBB integrity, models based on glia–endothelial co-culturing have been commonly used, and a significant improvement in TEER and a decrease in paracellular leakage have rewarded these endeavors.

Flow-based tridimensional models

Both short- and long-term changes occur in cerebral arterioles in response to intraluminal flow^{11,16–18}. Endothelial cells *in vivo* are continuously exposed to shear stress, which is generated by the flow of blood across their apical surfaces. Stanness *et al.* have recently developed a new *in vitro* model of the BBB characterized by a tridimensional, pronectin-coated hollow fiber structure that enables co-culturing of ECs with glia^{3,19–21}. In the hollow fiber apparatus, ECs are seeded intraluminally and are exposed to flow. Under these conditions, ECs develop a morphology that closely resembles the endothelial phenotype *in situ*, demonstrating that ECs grown with flow develop greater differentiation than after conventional culture¹¹. More recently, Stanness, Janigro and coworkers have reported the induction of BBB properties in ECs grown in hollow fibers in the presence of extraluminally seeded glia. This induction of a BBB-specific phenotype included low permeability to intraluminal potassium, negligible extravasation of proteins, and the expression of a glucose transporter. In addition, culturing of ECs with glia affected the overall morphology of the cells and induced the expression of BBB-specific ion channels^{19–21}.



The model system used for these studies results from a modification of a traditional cell culture system that is normally used for extensive culturing of non-EC cells. The general design of the hollow fiber apparatus is derived from attempts to develop a 'cell factory' (Fig. 1). Cell culture on hollow fibers was first described by Knazek and colleagues²² and has since been extensively exploited for mass production of rare cell types, antibody production and modeling of organ-like structures such as the BBB. Ott *et al.* used a hollow fiber cell culture apparatus for studies of flow-mediated effects on EC growth. Stanness and colleagues further developed this system by allowing combinations of intra- and extraluminal growth to study the effects of glia on EC.

The dynamic *in vitro* BBB model (DIV-BBB) features a plastic support that contains a variable number of artificial capillaries (AC) (330 μm cross diameter with a lumen of approximately 70 cm^2). These capillaries bear 0.5 μm transcapillary pores that allow free diffusion of solutes from the extraluminal compartment to the intraluminal space and *vice versa*. The capillaries are intraluminally perfused at various shear stress rates by pulsatile flow. It was shown that induction of BBB-like characteristics occurs following prolonged co-culture of glia and bovine aortic ECs, and that glia can induce the expression of BBB-specific ion channel proteins in non-BBB ECs^{19,23}. Interestingly, genetically altered astrocytes lacking intermediate filaments were not capable of these induction properties^{3,24}.

Drug passage across the DIV-BBB

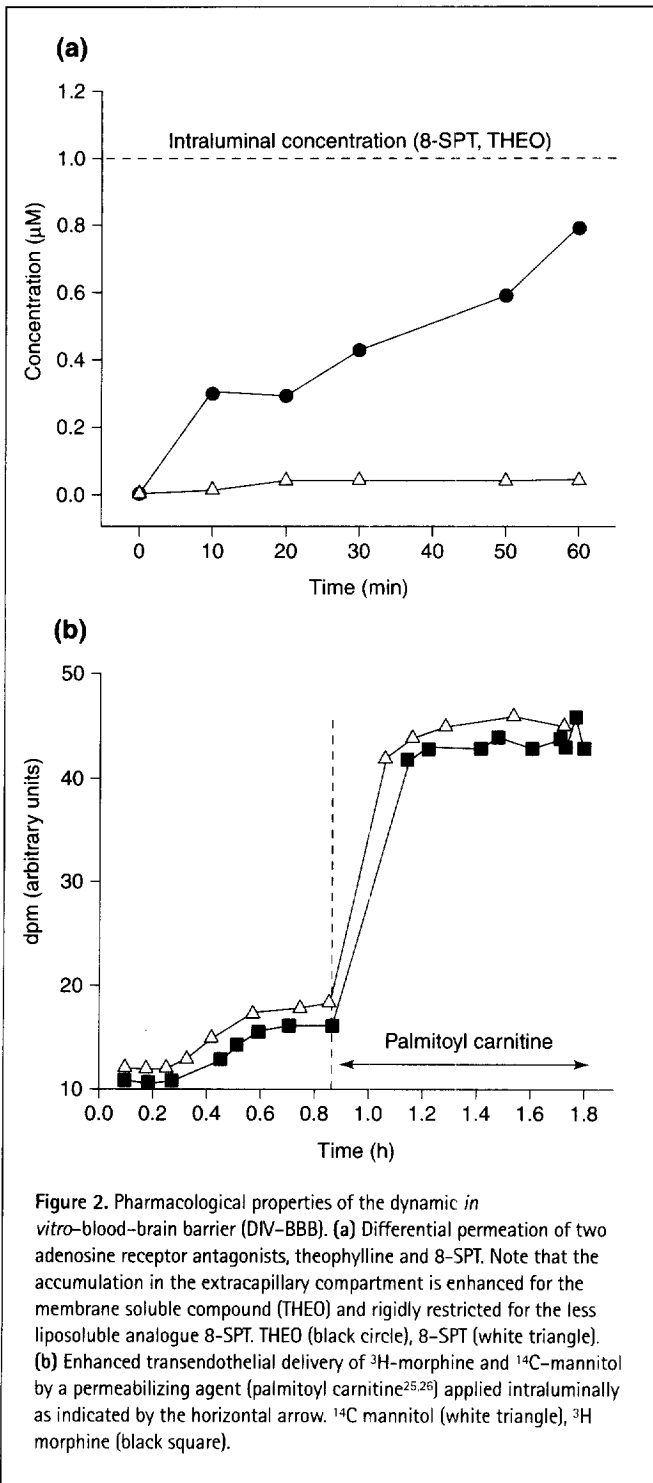
Drug and solute transport from the blood into the brain is limited by the membrane permeability of the EC, its intracellular metabolism and the lack of a major paracellular 'leak' route. Because of the absence of transcellular 'pores' or fenestrations, extravasation of drugs into the CNS depends largely – but not exclusively – on the lipid solubility of the drug and on the presence of drug transporters. The development of new drug delivery systems to bypass the BBB, as well as the design of systemically targeted drugs that do not cross the BBB, depends heavily on the availability of a biological model of the BBB that allows

for a careful, yet rapid, quantification of drug accumulation in the CNS following systemic administration. One of the limitations of the available *in vitro* models has been the inability to reproduce simultaneously both the permeability properties of EC membrane and the expression of transport systems. This is particularly important when dealing with pharmacokinetic or pharmacodistribution studies, in which both lipophilicity and co-transport/facilitated transport become important.

When the DIV-BBB was exposed to intraluminal substances that are known to feature a reluctance to permeate through the *in vivo* BBB [such as morphine, sucrose or mannitol, Fig. 2(b)], the calculated transendothelial permeability values were consistent with those reported from *in vivo* studies. In addition, exposure to 'permeabilizing agents', such as palmitoyl carnitine, increased extravasation in a manner that was identical to that reported *in vivo*^{25,26}. Furthermore, a permeability ratio of >100 between theophylline and sucrose has been reported *in vivo* and similar results were obtained in the DIV-BBB²¹. Finally, theophylline, but not its less lipid-soluble analogue 8-SPT, migrated rapidly across the endothelium [Fig. 2(a)]. Furthermore, the overall relationship between lipophilicity and permeability across the DIV-BBB was similar to that reported by others *in vivo*.

A further similarity between the selective drug permeability of the *in vivo* BBB and the *in vitro* model has been reported. Stanness and Janigro compared the permeability of polar amino acids that are transported across the BBB by stereospecific transporters. It was found that the biologically active L-isomer of aspartic acid was transported across the DIV-BBB at a significantly higher (>100) rate than its D-isomer counterpart. This suggested that this *in vitro* BBB model may be suitable for the study of the permeation of drugs that are normally accumulated in the CNS by virtue of a BBB- and stereo-specific transporter. When manipulations aimed at improving the permeation of substances were used, such as co-application of lipophilic emulsions, increased extravasation was observed, as previously documented *in situ*.

There are, however, obvious limitations to the DIV-BBB model and these will be addressed in future studies. These include:



- absence of blood cells;
- no influence of intraluminal pressure changes, which typically occur *in vivo*;
- lack of neuronal influences.

Recent developments have addressed at least one of these issues, and serotonergic neurons were successfully grown in

the abluminal compartment of the DIV-BBB. Preliminary experiments suggested that the presence of neuronal cells causes little, if any, effect on the functional properties of the DIV-BBB.

Is the BBB altered in neurological disorders?

A group of toxic agents and pathological conditions cause early changes in BBB function. These are mediated through direct effects on the ECs, but these changes are also usually associated with morphological changes in astrocytes. Brain tumors, for example, disrupt the glial sheath, which envelops the ECs, and CNS neoplasms are associated with increased capillary permeability. The capillary endothelium of tumor vessels is highly abnormal and expresses a range of fenestrated regions, vesicles, open junctions and fragmented basal lamina to a high degree, depending on tumor type and grade, which leads to a considerable increase in permeability of the tumor vascular bed. Tumors also stimulate the proliferation of abnormal capillaries by releasing angiogenic factors. Tumors therefore permit contrast enhancement on radiographic imaging studies (computerized tomography or magnetic resonance imaging) and may exhibit marked vasogenic edema. These findings may predict therapeutic success in the treatment of brain tumors by traditional, intravascular chemotherapy. The clinical reality however, is different, and malignant brain tumors are highly resistant to a variety of chemotherapeutics. This may be due to the fact that metastatic portions of the tumor are hidden behind a 'normal' or simply 'attenuated' BBB. Further studies are necessary in order to fully understand how tumor cells impact BBB function.

In an attempt to overcome the seemingly intractable nature of brain tumors, hyperosmolar solutions or analogues of the endogenous mediator bradykinin have been employed to improve the delivery of chemotherapeutic agents to the neoplastic cells. Future therapeutic strategies depend on an improved understanding of the mechanisms responsible for the induction and maintenance of the barrier, and modulation of the barrier under normal and pathological conditions. Furthermore, by co-culturing neuronal, glial and brain ECs with tumor cell lines, it may be possible to obtain important information on the efficacy of anti-neoplastic agents, while simultaneously monitoring the passage of these agents across the BBB and their potential neurotoxicity.

Requirement for *in vitro* models of pathologic BBB

There is increasing evidence that in many diseases of the CNS, the barrier dysfunction may be caused by the release, or activation of a cascade, of mediator substances from damaged or activated cells. The study of the conditions that increase BBB permeability has led to an improvement in the understanding of the mechanisms that maintain and modulate the barrier and the search for agents that can be used to open the barrier for therapeutic purposes. The role of the BBB in the evolution of

viral and bacterial CNS diseases remains incompletely defined and is currently being explored both *in vivo* and *in vitro* (for examples see Refs 27,28). Barrier dysfunction that is secondary to viral or bacterial pathogens may exacerbate the severity of the neurological injury, while an intact barrier may obstruct recovery from disease by delaying the entry of immune complexes or therapeutic agents into the infected CNS.

As shown in Box 1, perturbations of the BBB have been reported in a wide variety of CNS diseases, including infectious diseases such as AIDS or syphilis. In post-mortems performed on the brains of AIDS patients, diffuse leakage of serum proteins into the brain parenchyma is commonly observed. Because brain vessels are normally impermeable to serum proteins, due to the presence of tight junctions, it has been suggested that abnormal vascular permeability may be an important process in the development of HIV-induced CNS dysfunction. Infection of brain ECs may cause perturbations in BBB function, allowing toxic substances to cross into the normally inaccessible CNS. Alternatively, HIV-infected cells may travel through a damaged BBB and thereby introduce virus and/or viral proteins into the CNS.

Viral or bacterial infection of the brain endothelium could result in BBB perturbations via disruption of tight junctions or by an alteration of selective transport mechanisms. The subsequent enhanced vascular permeability could result in the entry into the CNS of neurotoxic cytokines, ions and metabolites from the periphery, which would contribute to neuronal dysfunction. Alternatively, infection of the BBB may change cell-surface expression of adhesion molecules, which could mediate recruitment and extravasation of activated macrophages. An additional possibility concerns the development of mutated forms of the offending pathogen, as recently shown for simian immunodeficiency virus (SVI) by Nelson and coworkers²⁹. By using the DIV-BBB model in its simian configuration (monkey brain endothelium and astrocytes), the authors demonstrated that, at least *in vitro*, the brain endothelial barrier may act as a reservoir for the virus, and allow persistent, long-lasting infection that results in the mutation of the virus to a clearly different phenotype. These results demonstrate that predictive experiments on the efficacy of CNS antivirals/antibacterials must be performed in 'disease' models of the BBB to ensure that there is targeting of a broad spectrum of infectious phenotypes (such as *in vivo*). To this end, it is also important to stress the importance of 'humanized' models, as numerous pathological conditions could not be faithfully reproduced in rodent-based or cell line-derived BBB models.

Modern understanding of brain pathophysiology has led to the provocative thought that many diseases of the CNS are associated with a failure of BBB structural integrity or function. Thus, altered BBB permeability is commonly observed during ischemia, inflammation, trauma, neoplasia, hypertension, dementia and epilepsy. The extravasation of plasma proteins with

BBB dysfunction may occur through a number of different transcellular or paracellular routes, including altered tight junctions, induction of fluid-phase or nonspecific pinocytosis and transcytosis, formation of transendothelial channels, or by disruption of the EC membrane. These pathways are not mutually exclusive. Irrespective of whether the BBB disruption is the main pathogenic factor or an inevitable consequence of the disease itself, understanding of the cellular mechanisms that cause disruption of the BBB is limited. This situation is due, in part, to the lack of comprehensive models of BBB. Any such models must reproduce the salient features of normal BBB (Box 2),

Box 1. CNS disease and the blood-brain barrier (BBB): implications for drug delivery (increased/decreased permeability) and therapeutic strategies (does the BBB play a role in the etiology of the disease?). Putative etiologic agents or cells are listed in parentheses

Neoplasia

Brain tumors (histamine, tissue necrosis factor, interferons, interleukins, permeable tumor vessels)

Meningiomas (vascular endothelial growth factor)

Vascular

Ischemia, hypoxia (glutamate, free radicals, vasodilation, lactic acidosis, prostaglandin, glial dysfunction)

Hypertension (mechanical damage to endothelium, free radicals, vasopressin, angiotensin)

Subarachnoid hemorrhage (complement system C3a, endothelial cell damage, vasospasm)

Arteriovenous malformations (endothelial damage due to ischemia and high flow state)

Migraines (*serotonin*)

X-irradiation (endothelial damage)

Trauma

Open and closed head injury (intracranial hypertension, endothelial disruption, vascular spasm and loss of cerebral autoregulation)

Brain edema

Vasogenic (endothelial damage, intracranial hypertension, arachidonic acid metabolites, histamine, oxygen free radicals, polyamines)

Cytotoxic

Metabolic

Diabetes (hyperglycemia, ischemia)

Toxins: lead, aluminum, mercury, DMSO (*endothelial damage*)

Epilepsy

Seizures (glutamate, glial dysfunction following neuronal activation, hypertension)

Inflammation

Multiple sclerosis/experimental allergic encephalomyelitis

Meningitis: bacterial, viral, fungal (bradykinin, ATP, histamine, serotonin, interleukins)

Box 2. Physiological and functional properties of the *in situ* blood-brain barrier

BBB specific markers	+++
Inductive influences from glia	Mandatory
Tight junctions	+++
Transendothelial resistance	1500 ohms/cm ²
Sucrose permeability	Low (<10 ⁻⁷ cm sec ⁻¹)
K1 permeability	Low
Exposure to flow	Luminal membrane
Polarized transporters	Ubiquitous (for example, K ⁺ , amino acids)
Stereoselective transport	Glucose, amino acids

Advantages of the dynamic *in vitro*-blood-brain barrier

- Use of cultured cells
 - Primary cultures
 - Transformed cells/cell lines
- Simultaneous measurement of intra- and extraluminal concentration of drugs
- Exposure of intraluminally seeded cells to laminar flow/shear stress
- Permeability properties similar to *in situ* BBB
 - High electrical resistance
 - Low permeability to ¹⁴C sucrose
 - Expression of segregated ion transporters
 - Stereoselectivity of amino acid transport
 - Physiological mechanism of glucose transport
- Possibility of long-term (months) studies
- Use of human tissue and human disease models (such as AIDS)
- Presence of drug extrusion mechanisms

while allowing for manipulations aimed at mimicking the disease process.

It is important to remember that the original studies on BBB in health and disease have used a variety of *in vivo* models, in which anesthetized animals underwent surgical procedures that allow simultaneous measurements of intra- and abluminal concentrations of drugs or endogenous substances (for examples, see the discussion in Refs 5,8,10). By considering *in vivo* data against modeling attempts, it becomes apparent that, although isolated cultured cells provide useful insight into the mechanism of BBB ontogenesis and physiological development, a meticulous experiment should be designed to include both observations gathered *in vitro* as well as data from intact animals.

It is frequently assumed that the ideal conditions to test BBB permeability to putative neurotherapeutics consist of experiments on 'tight' and 'physiologic' BBB models. Because of the increasing index of neurological disorders characterized by deficient expression of diverse BBB markers, one might expect that in the future it will become imperative to perform these studies on 'diseased' BBB models. This appears particularly

important when attempting to exploit BBB-specific mechanisms (such as GLUT1 or a particular receptor for a virus) for drug delivery to the brain: it is possible that these physiologic phenotypes might be altered or altogether missing in the diseased brain.

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