Chapter 8
In Vitro Models of CNS Barriers

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Abstract In vitro models of the blood-brain barrier provide valuable mechanistic information and useful assay systems for drug discovery and delivery. However, it is important to take into account issues including species differences and to what extent features of the in vivo BBB are retained in cell culture. The history and applications of a primary cells, immortalized cell lines, and stem cell-derived BBB models are reviewed, with evaluation of their strengths and weaknesses, in selecting and optimizing a suitable model for particular applications. Understanding of the unstirred water layers gives insights into the "intrinsic permeability" of the membrane, and proteomic and transcriptomic studies have expanded the characterization of the barrier function. Technologies to derive brain endothelium from human stem cells create 3D models of the neurovascular unit, and miniaturize "organ-on-a-chip" flow systems give great promise for the future. All these technologies are crucial to translate BBB research to viable treatment options for patients.

Keywords Blood-brain barrier · In vitro models · Endothelia · Astrocyte · Pericyte · TEER · Primary cells · bEND.3 · hCMEC/D3 · iPSC · IVIVC

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E. C. M. de Lange et al. (eds.), *Drug Delivery to the Brain*, AAPS Advances in the Pharmaceutical Sciences Series 33, https://doi.org/10.1007/978-3-030-88773-5_8

8.1 Introduction

From the earliest demonstration of restricted exchange between the blood and the brain (Ehrlich 1885) leading to the modern understanding of the blood-CNS barriers, animal experiments and clinical observations have provided valuable information about the physiology and pathology of the barrier layers. However, obtaining mechanistic information from such studies at the cellular and molecular level is complex and time-consuming, and it is often difficult to obtain sufficient spatial and temporal resolution. The situation was dramatically improved by the introduction of in vitro methods (reviewed in Joó 1992).

8.1.1 Background and Early History

The first successful isolation of cerebral microvessels (Siakotos and Rouser 1969; Joó and Karnushina 1973) prepared the way for development of in vitro models of the blood-brain barrier (BBB), which have contributed to current understanding of its physiology, pharmacology, and pathophysiology (reviewed in Joó 1992). Methods have also been developed for in vitro models of the choroid plexus and of the arachnoid epithelium (blood-CSF barrier, BCSFB). However, this proliferation of in vitro models and techniques causes problems for attempts at comparison between models and transferability of results obtained with different models and makes it hard for scientists entering the field to select an optimal model for their particular interests. This chapter gives an overview of the current status of the most widely used in vitro CNS barrier models, with an update on an earlier review (Abbott et al. 2014; Reichel et al. 2003), and offers guidance in model selection for specific applications, including permeability assay for drugs and "new chemical entities" (NCEs).

Isolated brain microvessels were the first model system for studying the BBB in vitro, offering new opportunities to investigate physiological and pathological processes at the cellular, subcellular, and molecular level (Pardridge 1998). A new generation of in vitro models emerged with the first successful isolation of viable brain endothelial cells (BECs), which could be maintained in cell culture (Brendel et al. 1974; Panula et al. 1978; Bowman et al. 1981; see Joo 1992). There followed a number of advances which allowed improved isolation of endothelial cells from brain capillaries with minimal contamination from cells of arterioles and venules, both improving the "barrier phenotype" of the endothelial monolayer and minimizing the contamination by smooth muscle cells, pericytes, and glia (Krämer et al. 2001). The first successful growth of endothelial cells on filters (Fig. 8.1a) allowed measurement of transendothelial permeability, and adopting technology developed for epithelia (Grasset et al. 1984) allowed monitoring of transendothelial electrical resistance (TEER) as a measure of tightness to small ions (Rutten et al. 1987; Hart et al. 1987). Many of the techniques for understanding ways to improve the yield,

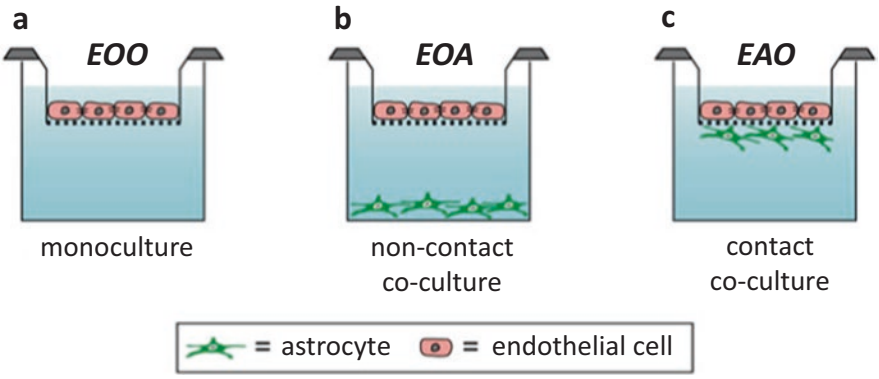

Fig. 8.1 Configurations for brain endothelial cell-astrocyte co-culture models. The three-letter label indicates cell location, in the following order: on the top of filter, on the underside of filter, and in the base of well. Thus panel (**a**) shows a typical monolayer culture with endothelial cells E on top of the filter and no other cell types present; hence EOO, (**b**) shows noncontact co-culture with astrocytes A or mixed glia in the base of the well (EOA) and (**c**) shows "contact" (note that depending on the size of the filter pores and time in co-culture, the glia may or may not actually send fine processes through the filter to contact the endothelial cells) co-culture with astrocytes growing on the underside of the filter, with no cells in the base of the well (EAO). (Redrawn by R Thorne, based on Nakagawa et al. 2009, with permission)

viability, and expression of differentiated phenotype benefited from parallel developments in growing epithelial cells especially Caco-2 (Wilson 1990).

Protocols for isolating and maintaining brain endothelial cells have been described for a large number of species including mouse, rat, cow, sheep, pig, monkey, and human, typically producing confluent cell monolayers after about 9 days in culture (Garberg 1998; Deli et al. 2005). However, with passage, cultured BECs tend to show diminished characteristics of the in vivo BBB, e.g., tight junctional complexity, specific transporters, enzymes, and vesicular transport, reverting toward the "default" non-brain endothelial phenotype characteristic of early BBB development (Daneman et al. 2010b). DeBault and Cancilla (1980) first reported that many of these BBB features can be at least partly reestablished by co-culturing the BECs with astrocytes in arrangements allowing either direct contact or noncontact humoral exchange. Co-cultures with astrocytes followed with improved BBB phenotype (Fig. 8.1b, c) (Dehouck et al. 1990; Rubin et al. 1991; Kasa et al. 1991; see Cecchelli et al. 1999). It should be noted that a complication of contact co-culture (Fig. 8.1c) during transport studies is the continuing presence of the astrocytes. Lipophilic compounds in particular may become trapped in the astrocytes, and many drugs are metabolized by enzymes highly expressed in the astrocyte layer (Dutheil et al. 2010). However, it is argued that the close association of endothelium and astrocytes mimics that in vivo, hence providing a good model for studying flux across the "combined barrier."

During the next stage of development, some of the more sophisticated primary cultured models became so complex to prepare and maintain that they were not

practical for routine assays; this was at least partly the motivation for the generation of much simpler models employing immortalized cell lines. The ready availability of molecular biological techniques led to creation of immortalized and transfected CNS barrier cell line models (Reichel et al. 2003 Deli et al. 2005). However, unlike the well-accepted Caco-2 cell line employed for studies of intestinal absorption, or Madin-Darby canine kidney (MDCK) cells used as reliable epithelial models, there were no uniformly satisfactory cell line models for studying the BBB and other CNS barriers in vitro, mainly because of the poor development of tight junctions and hence generation of models on filters that were too leaky for study of transendothelial or transepithelial permeation. Most recently, BBB in vitro models have been derived from human stem cells (Lippmann et al. 2012; Cecchelli et al. 2014). These successfully generate very tight monolayers with endothelial-like phenotype (Le Roux et al. 2019), although they also express epithelial-like adhesion molecules and transporters which may complicate interpretation (Lu et al. 2021). Attempts to reintroduce lost BBB features, or silence non-BBB features in immortalized or stem cell models by means of transfection/transduction, are a promising prospect with mixed success so far (Gericke et al. 2020; Lu et al. 2021) but with great future potential. Rather, molecular techniques allowing more subtle manipulation of cells for experimental purposes (e.g., to introduce imaging tracers, Huber et al. 2012) are proving practical and popular.

In vitro systems generally do not express fully the in vivo properties of the BBB, so specific modifications continued to be introduced to study particular aspects of BBB function. As the in vitro systems developed differed with respect to isolation procedures, cell culture conditions and configuration (mono-/co-culture), and the cell type (origin and species), attempts were made in a European Union Concerted Action Programme (1993–1997) to standardize the most popular models to facilitate comparison of the data collated from different laboratories (Garberg 1998; de Boer and Sutanto 1997). ECVAM, European Centre for Validation of Alternative Methods, also sponsored comparison between different in vitro BBB and epithelial models as CNS drug permeability assay systems (Garberg et al. 2005; see also Avdeef 2011). However, since no consensus emerged as to the "best model," most groups have continued to improve, optimize, and extend the range of applications of the models they selected or developed for historical and practical reasons. Indeed, over the last 15 years, significant progress has been made to the point that scientists new to the field have range of good and practical options (see Table 8.2) and can make informed choices. Some key landmarks in development of in vitro CNS barrier models are shown in Table 8.1.

8.1.2 Criteria for Useful In Vitro CNS Barrier Models

The ideal in vitro CNS barrier model would preserve in a reproducible way all the features of the in vivo equivalent and be straightforward and inexpensive to prepare. The features to reproduce would include all aspects of the "physical, transport, and

Table 8.1 Landmarks in development of in vitro BBB models

Landmark advance	References
Isolation of brain microvessels	Siakotos and Rouser (1969), Joó and Karnushina (1973)
Growth of brain endothelial cells in culture	Panula et al. (1978), Bowman et al. (1981)
Growth of brain endothelial cells on filters, TEER measurement (bovine, human)	Rutten et al. (1987), Hart et al. (1987)
Development of immortalized cell line models mouse, rat, bovine, porcine, human	1988 onwards; see text and Table 8.2
Clonal bovine brain endothelial cell culture to avoid contaminating pericytes, co-culture with astrocytes (base of well) TEER >600 Ω.cm^2	Dehouck et al. (1990)
Addition of differentiating factors to medium to improve BBB phenotype (bovine, porcine)	Rubin et al. (1991) (CPT-cAMP), Hoheisel et al. (1998) (hydrocortisone)
"Dynamic" BBB model with intraluminal flow (DIV-BBB)	Stanness et al. (1996, 1997)
Tight porcine brain endothelial cell layer without astrocytes, TEER 700 (up to 1,500) Ω.cm^2	Franke et al. (1999, 2000)
Further option for co-culture—astrocytes on the underside of filter, tighter layer (bovine)	Gaillard and de Boer (2000)
Confocal microscopy method for transport studies in isolated brain microvessels	Miller et al. (2000)
Conditionally immortalized rat, mouse cell lines from the brain and retina endothelium, choroid plexus	Terasaki and Hosoya (2001)
First BBB genomics screen, isolated rat brain microvessels	Li et al. (2001, 2002)
Addition of puromycin to kill contaminating pericytes (rat)	Perrière et al. (2005)
Introduction of hCMEC/D3 human immortalized brain endothelial cell line	Weksler et al. (2005)
Quantitative proteomics of brain endothelium	Kamiie et al. (2008)
Tri-culture models—endothelium, pericytes, astrocytes	Nakagawa et al. (2009)
Transcriptome analysis of purified brain endothelium	Daneman et al. (2010a)
Method to measure and correct for unstirred water layers, paracellular permeability for cells on filters, allowing improved in vitro-in vivo correlation (IVIVC)	Avdeef (2011)
Human stem cell-derived BBB models introduced	Lippmann et al. (2012), Cecchelli et al. (2014)
Microfluidic BBB model prototypes	Booth and Kim (2012), Prabhakarpandian et al. (2013), Griep et al. (2013)
3D spheroid development. Self-assembly of endothelial sphere surrounding a monolayer of pericytes and astrocyte core	Urich et al. (2013)
"Organ-on-a-chip" technology. Miniaturized, 3D microfluidic flow system for co-culture with neurons and astrocytes	Brown et al. (2015) and Adriani et al. (2017)

Table 8.2 The most widely used immortalized cell lines and primary cell models of the BBB

Immortalized cell line	Species, transfection	1st publication	Recent references	Number of citations to March 2021	Number of citations 2019–2020
bEND.3†	Mouse (3)	Williams et al. (1989); Montesano et al. (1990)	Zhang et al. (2021); Wainwright et al. (2020); *García-Salvador et al. (2020)	595	145
hCMEC/D3†	Human (5)	Weksler et al. (2005)	Laksitorini et al. (2020); Fatima et al. (2020); *Veszelka et al. (2018)	434	136
iPSC, BLEC	Human stem cell derived	Lippmann et al. (iPSC) *2014; Cecchelli et al. (BLEC) 2014	Li et al. (2021); Nishihara et al. (2020); *Raut et al. (2021)	111	55
RBE4	Rat (2)	Roux et al. (1994)	Baumann et al. (2021); Sadeghzadeh et al. (2020);	186	11
bEND5†	Mouse (3)	Wagner and Risau (1994)	Devraj et al. (2020)	27	5
TR-iBRB2	Rat retina (4)	Hosoya et al. (2001)	Akanuma et al. (2018)	50	2
cEND†	Mouse (3)	Förster et al. (2005)	Ittner et al. (2020)	18	1
GP8.3	Rat (1)	Greenwood et al. (1996)	Veszelka et al. (2018)	27	1
GPNT†	Rat (1)	Régina et al. (1999)	Regan et al. (2021)	20	1
TR-BBB13	Rat (4)	Hosoya et al. (2000)	Tachikawa et al. (2020)	14	1
MBEC4	Mouse (1)	Shirai et al. (1994)	Mizutani et al. (2016)	44	0
HBMEC/ciβ	Human (4)	Kamiichi et al. (2012)	Masuda et al. (2019)	3	1
Primary cells	**Species**	**1st publication**	**Recent references**		**Number of citations 2019–2020**
	Mouse†	DeBault et al. (1979); Hansson et al. (1980)	Liu et al. (2020); Puscas et al. (2019); *Wuest et al. (2013)		36

(continued)

Table 8.2 (continued)

	Rat†	Bowman et al. (1981)	Luo et al. (2020); Ohshima et al. (2019); *Watson et al. (2013)		31
	Human†	Dorovini-Zis et al. (1991)	Nascimento Conde et al. (2020); Devraj et al. (2020); *Li et al. (2015)		21
	Porcine	Mischeck et al. (1989)	Woods et al. (2020); Di Marco et al. (2019); *Gericke et al (2019)		14
	Bovine	Dorovini-Zis et al. (1984)	Goldeman et al. (2020); Kristensen et al. (2020)		12

Transfection vectors/method: (1) SV40 large T antigen; (2) adenovirus El A gene; (3) Polyon, virus middle T antigen; (4) temperature-sensitive SV40 large T antigen; (5) sequential lentiviral transduction of hTERT and SV40 large T antigen. †Commercially available. *Source data for Fig. 8.3 showing TEER vs. permeability

enzymatic barrier" functions outlined in Chap. 1 and, where relevant, also their immunological features. Replicating the in vivo environment can retain or upregulate BBB features, e.g., by providing luminal medium flow to mimic blood flow shear stress; co-culturing with multiple cells of the neurovascular unit including neurons, pericytes, and astrocytes; and culturing in 3D capillary-like tubes (Booth and Kim 2012; Adriani et al. 2017). However, in the context of this volume, the models should also provide easy to use, readily available and reproducible assay tools for the reliable prediction of the penetration of compounds including drugs into the CNS in relation to both the route and rate of brain entry.

Thus far, no single BBB or BCSFB model fulfills these stringent requirements. However, satisfactory results may be obtained with models expressing the most critical features of the BBB or BCSFB in vivo that are relevant for the particular interest of the study. This means that it is important that users undertake basic model characterization to include the specific BBB feature(s) for which the model is then applied.

8.1.3 The Physical Barrier and Tight Junctions: Monitoring CNS Barrier Tightness In Vitro

The expression of functional tight junctions between the BECs is one of the most critical features due to their consequences for the function of the BBB. In the in vivo BBB, complex and extensive tight junctions contribute significantly to the control

over CNS ion and molecular penetration. This is achieved by (1) very severe restriction of the paracellular pathway, (2) limiting flux of permeant molecules to transendothelial pathways, (3) associated expression of specific carrier systems for hydrophilic solutes essential for the brain (e.g., nutrients), and (4) permitting polarized expression of receptors, transporters, and enzymes at either the luminal or abluminal cell surface allowing the BBB to act as a truly dynamic interface between the body periphery (blood) and the central compartment (brain), capable of vectorial transport of certain solutes.

As discussed in Chap. 1, the tight junctions of the choroid plexus epithelium and arachnoid express different claudins than those of brain endothelium and are leakier than those of the BBB; however, their presence in the epithelial barrier layers has a similar effect on the properties of these epithelia, e.g., in polarization of function and regulation of transepithelial transport.

8.1.3.1 Methods to Measure Barrier Permeability and TEER

In vitro models to be used for transendothelial/transepithelial drug permeation studies need to have sufficiently restrictive tight junctions to impede paracellular permeation, mimicking the in vivo situation. Paracellular permeability can be assessed using inert extracellular tracers (Avdeef 2011, 2012). For tighter layers, small tracer molecules can be used, such as radiolabelled sucrose (MW 342, hydrodynamic radius r: 4.6 Å or 0.46 nm) or mannitol (MW 182, r 3.6 Å), or fluorescent markers such as Lucifer yellow (LY; MW 443, r 4.2 Å) or sodium fluorescein (MW 376, r 4.5 Å). For leakier layers, larger tracers used such as inulin, dextrans, and serum albumin are used to characterize paracellular pathways. However, the use of these tracers is labor-intensive and time-consuming, inevitably involving additional assays and analytical delays, and has poor time resolution, and fluorescent tracers may interfere with analysis or permeation of, for example, fluorescent substrates of membrane transporters.

For less invasive monitoring, measurement of transendothelial/epithelial electrical resistance (TEER) is simpler, gives a real-time readout, and has a variety of applications: (1) to monitor the status of the barrier layer, especially for cells grown on opaque filters where visual inspection of confluence is not possible; (2) to determine the culture day on which optimum tightness is reached for experiments; (3) in quality control of cells grown on filters, establishing the baseline permeability of cell monolayers on individual filters to allow exclusion of poor monolayers that fall below a satisfactory threshold tightness; and (4) to follow changes in resistance over time, e.g., to follow the effects of particular growth conditions or a drug or pharmacological agent on barrier integrity and tight junction function.

Before measuring TEER, it is important to know that choice of filter membrane will impact TEER measurement in several ways, regardless of the tightness of cell monolayer. First, smaller filters will give lower TEER due to the edge "effect." Cells cannot make a tight junction at the circumference of the filter where they meet the polystyrene at the edge, and this contributes to paracellular leak, particularly in

filters with a small surface area relative to the circumference (e.g., in 24-well formats; Stone et al. 2019). Second, clear Transwell filters have fewer membrane pores per cm^2 compared to translucent filters, regardless of the pore size (e.g., Falcon and Costar "Snapwell" Transwell insert pore densities; clear ~ $1 \times 10^6/cm^2$; translucent ~ $1 \times 10^8/cm^2$), which results in increased TEER not entirely mitigated by subtraction of TEER across a "blank" filter. Third, increasing pore size above 1 μm allows cells to migrate through the pores from the apical side to form a second layer of cells on the underside of the filter, resulting in increased TEER (Wuest et al. 2013).

Two main types of TEER system are used (Fig. 8.2; Benson et al. 2013). The first and simplest is the voltohmmeter (VO) (Fig. 8.2a), where a pair of current and voltage electrodes in "chopstick" array is used. In the second, more recently developed instruments use the method of impedance spectroscopy (IS) (Fig. 8.2b). Permit monitoring of both TEER across cell layers and IS allows continuous analysis over hours to days and also gives information about the electrical capacitance which can reveal additional features of the barrier properties such as cell shape and the degree of cell-substrate adhesion. The earliest IS devices involved growing cells on solid microstructured electrodes, so these systems were not suitable for use in association with drug permeability screening. More recently developed systems permit use of cells grown on porous filters and simultaneous monitoring of multiple filters, e.g., in a 12- or 24-well format.

8.1.3.2 TEER Measurement Based on Ohm's Law: V = IR (Voltage = Current × Resistance)

In the most widely used VO applications (Fig. 8.2a), such as the WPI (World Precision Instruments) "EVOM" system (and Millipore/Millicell equivalent), an AC (alternating current) square wave, here at 12.5 Hz, is passed between voltage electrodes in either side of the cell layer, the resulting current is measured, and the ohmic resistance R is derived. When multiplied by the surface area of the filter membrane, this gives TEER in $\Omega.cm^2$. A few papers in the literature give the units of TEER as "Ω/cm^2" which is incorrect, and this suggests that the authors do not fully understand the theory or methodology. An AC voltage source is preferred over DC as the latter can have polarizing effects on the electrodes or damage the cells. Earlier designs of chopstick electrode pairs (e.g., WPI STX2) were flexible, making it difficult to place the electrodes at a constant distance apart. Recent improvements in design give fixed electrode spacing (e.g., STX100C) and hence better reproducibility. The "Endohm" chamber system with large plate electrodes to fit in the filter cup (above) and the well (below) the cells on the filter, can sample a larger area of membrane including the more uniform central area and can give more reproducible readings (Cohen-Kashi Malina et al. 2009; Helms et al. 2010, 2012; Patabendige et al. 2013a, b); however, the "plunger" action of inserting the upper electrode can disturb the cells, particularly brain endothelial cells, which are much thinner and more fragile than the CNS barrier epithelial cells.

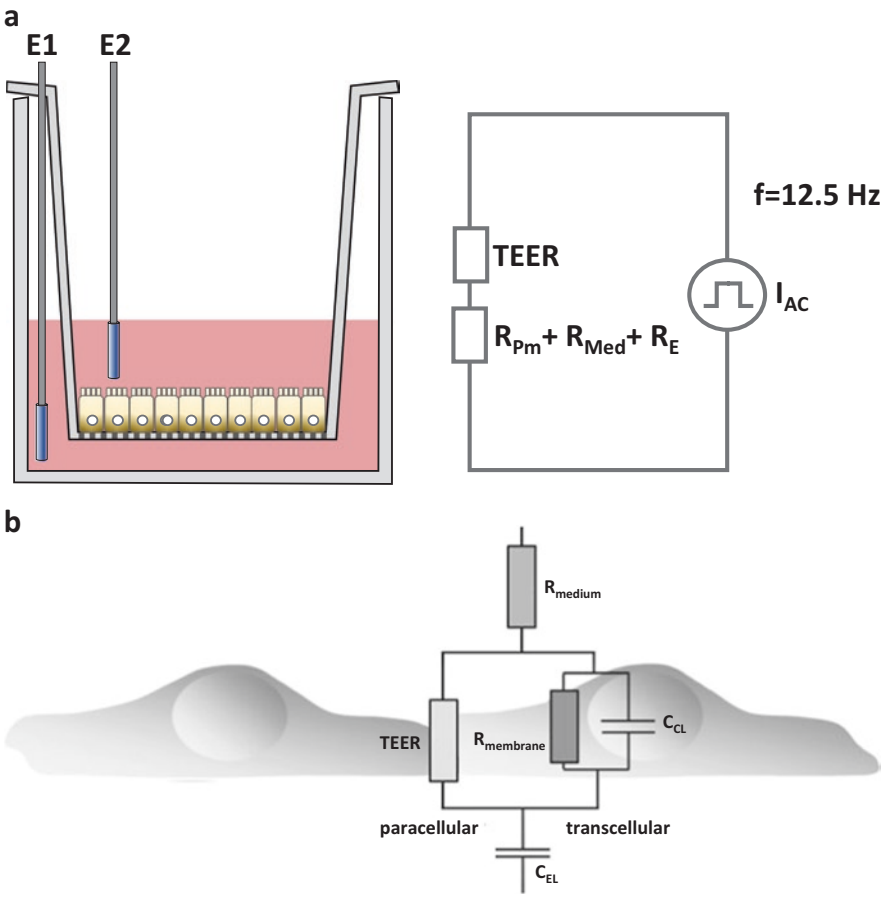

Fig. 8.2 Methods to measure TEER. (**a**) Resistance measurement in voltohmmeter (VO) system using "chopstick" electrodes. The electrodes (E1, E2) in either side of the cell monolayer on the porous filter are used to determine the electrical resistance. The ohmic resistance across the cell layer (TEER), the cell culture medium in the upper and lower compartments (R_{Med}), the membrane of the filter inserts (R_{Pm}), and electrode-medium interface (R_E) all contribute to the total electrical resistance. I_{AC}, alternating square wave current. (**b**) Measurement of TEER and capacitance in impedance spectroscopy (IS) system. Equivalent circuit diagram showing the contribution of the transcellular and paracellular pathways to the total impedance, Z, of the cellular system. *TEER* transendothelial electrical resistance, C_{EL} capacitance of the electrodes, C_{CL} capacitance of the cell layer, R_{medium} ohmic resistance of the medium, and $R_{membrane}$ ohmic resistance of the cell membranes. For tight endothelia and epithelia, TEER is dominated by the transcellular pathway. TEER is determined from the circuit analysis using Z measured at different frequencies of alternating current. (From Benson et al. 2013, with permission)

8.1.3.3 Impedance Spectroscopy Systems

An IS device (Fig. 8.2b) that has proven reliable in the context of BBB and choroid plexus epithelial (CPE) models is the "cellZscope" system (nanoAnalytics), available in different formats capable of accommodating 6, 12, or 24 filter inserts and

giving continuous readout of TEER (Benson et al. 2013). The system is computer-controlled, and TEER and capacitance are derived from an electric equivalent circuit model within the software. There is an optimum frequency range appropriate for deriving TEER and capacitance. One drawback of this system is the indirect method for calculating TEER, which relies on the use of the equivalent circuit and certain assumptions about the way current will flow through the system at different frequencies. A nanoAnalytics technical note comparing TEER measured with the cellZscope system and with chopstick electrodes shows good correspondence when the system parameters are set correctly, in particular when impedance at low frequencies is used (f<1kHz; Cacopardo et al. 2019). However, there are some discrepancies in the impedance literature measuring TEER across cultured choroid plexus epithelial (CPE) cells. Wegener et al. (1996, 2000) grew porcine CPE cells on gold film electrodes and recorded TEER 100–150 Ω.cm^2, rising to 210 Ω.cm^2 in the presence of the differentiating agent 250 uM CPT-cAMP, while other studies reported TEER >1500 Ω.cm^2 in serum-free medium (reviewed in Angelow et al. 2004). Using a VO device, Strazielle and Ghersi-Egea (1999) recorded 187 Ω.cm^2 in primary rat CPE, while Baehr et al. (2006) reported 100–150 Ω.cm^2 in pig choroid plexus and commented this would be equivalent to ~600 Ω.cm^2 in an impedance system. Using a VO system with a stable continuous subcultivatable porcine CPE cell line, Schroten et al. (2012) reported TEER >600 Ω.cm^2. In general, the values up to ~600 Ω.cm^2 fit better with evidence for leakier tight junctions in CPE than BBB (Bouldin and Krigman 1975), but it is clear that more "side-by-side" comparisons of VO and IS systems using a particular in vitro model would be helpful to clarify the situation.

8.1.3.4 Relation Between Permeability and TEER

Since 1990 steady progress has been made in the standard (flat filter) in vitro system, to the point where some of the best are able to reach a level of tightness approaching the in vivo BBB (>1000–2000 Ω.cm^2) which is essential for the ionic homeostasis of the brain interstitial fluid required for neuronal function. For assessing solute and drug transport across the BBB, the tighter the monolayer, the better the resolution (dynamic range) for distinguishing between transendothelial permeability and paracellular "leak." Dynamic range can be established experimentally from the permeability ratio between a high and low permeant compounds, e.g., propranolol vs. sucrose. High dynamic range gives better discrimination and rank-ordering of compounds with similar physical chemical properties within a series. However, even models with medium-range tightness are capable of providing adequate resolution for certain applications, particularly if the models show reproducible tightness reflected in consistent values for solute permeability. Recent improvements in understanding, separating, and correcting for the components of in vitro systems that affect cell permeation (unstirred water layer/aqueous boundary layer and porosity of paracellular pathway) also provide ways to determine the true transcellular endothelial permeability, P_c (Avdeef 2011, 2012).

TEER effectively measures the resistance to ion flow ("charge" transfer) across the cell layer, carried by the chief charge carriers in body fluids and physiological saline solutions, Na^+ and Cl^-. The *conductance* "*g*" is the reciprocal of resistance ($g = 1/R$) and is a combined measure of both the ionic permeability of the cell layer and the total number (concentration) of available ions. *Permeability* ($cm.s^{-1}$) is the ability of a solute (including ions) to move through a membrane channel or pore, i.e., is a measure of "mass" transfer and is a property of the membrane or cell layer. Hence conductance is related to permeability.

Traditionally BBB groups have measured either the apparent permeability of the monolayer (P_{app}) or the endothelial permeability P_e, corrected for permeability of the filter. Since TEER is inversely related to permeability, a plot of permeability vs. TEER will give a falling exponential curve. Measuring TEER and permeability of a paracellular marker (e.g., sucrose, mannitol, some small fluorescent tracers) on the same filter with an attached monolayer are useful ways of monitoring the status and reproducibility of the preparation, both for quality control and for experimental studies (Gaillard and de Boer 2000; Lohmann et al. 2002).

Where the monolayer properties including P_{app} are reproducible, a TEER above ~150 $\Omega.cm^2$ may be sufficient to ensure P_{app} for small- to medium-sized molecules is relatively independent of TEER, i.e., giving accurate values for P_{app} (Gaillard and de Boer 2000), or even lower TEER may be suitable to determine P_{app} of macromolecules (Wainwright et al. 2020). Indeed, many groups have adopted a quality threshold of 200–250 $\Omega.cm^2$ for permeability assays of small drug molecules, which is not easy to achieve in some primary and immortalized cell line BBB models (Fig. 8.3). Lohmann et al. (2002) using monocultured porcine brain endothelial cells and measuring TEER with an impedance system found TEER in the range 300–1500 $\Omega.cm^2$; P_e was quite variable at low TEER so they set a threshold of 600 $\Omega.cm^2$ for cells to be used for experiments. It is clear that the appropriate threshold should be selected for the particular cell model, TEER measuring system used, and type of study.

8.1.4 Barrier Features Related to Transporters, Enzymes, Transcytosis, and Immune Responses

As with TEER, reasonable compromises may also be made with other aspects of the BBB. Indeed, it is generally accepted that for a particular application, the model needs only to be characterized for those features which are both relevant and critical for the point of interest. For example, for an in vitro BBB system useful to screen small drug compounds for their CNS penetrability, the model needs to be sufficiently tight and should possess relevant polarized carrier and efflux systems in order to produce useful information. Similarly, for examination of transendothelial or transepithelial permeation of large molecules and nanocarrier systems where vesicular routes may be involved, it is important that the cell system chosen reflects the specialized features of such transport in the polarized in vivo barrier system.

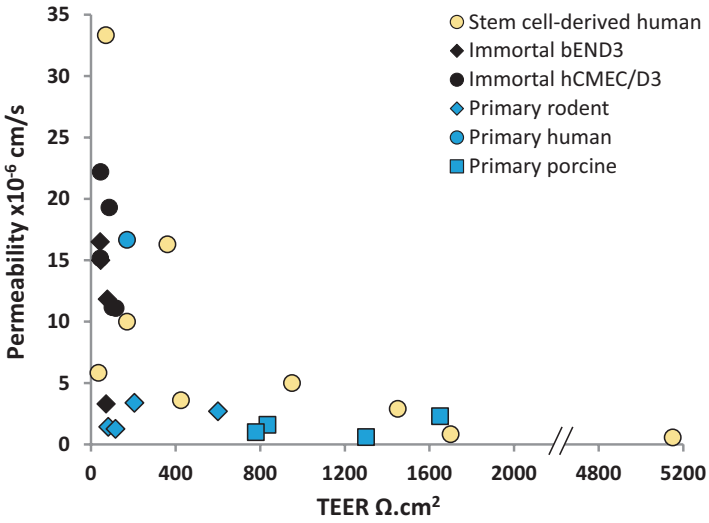

Fig. 8.3 Relationship between small molecule permeability and TEER in BBB in vitro models from multiple laboratories. Permeability is to one of sucrose (MW 342), sodium fluorescein (MW 376), mannitol (MW 182), or LY (MW 457), calculated as $P_{app} \times 10^{-6}$ cm/s or as $P_e \times 10^{-6}$ cm/s (P_e is essentially equivalent to P_{app} when the filter is freely permeable to the compound of interest). Data collated from recent models providing concurrent TEER and permeability data for their model. Cecchelli et al. (2014); García-Salvador (2020); Gericke et al. (2020); Helms et al. (2016); Le Roux et al. (2019); Li et al. (2015); Lippmann et al. (2014); Martins et al. (2016); Matsumoto et al. (2020); Patabendige et al. (2013b); Rand et al. (2021); Raut et al. (2021); Santa-Maria et al. (2021); Seok (2013); Smith et al. (2007); Veszelka et al. (2018); Watson et al. (2013); Wuest et al. (2013); Yamashita (2020); Zolotoff et al. (2020). Selected data sources are also referenced in Table 8.2

However, for many drug permeability projects, the model may not need to show the full complement of immunological responses which will only be necessary in those systems used to study the immune response of the CNS barriers. The existing in vitro model systems have very different levels of characterization and have generally been chosen for utility in a particular area of research interest.

8.2 Current Status: Overview of Current In Vitro BBB Models

Isolated brain capillaries can be used in suspension or fixed onto glass slides. By contrast, all cell-based systems require specific growth surface coatings and cell culture media for growing BECs. Although the cell preparations and culture conditions are all based on the same principle, in order to obtain functional in vitro BBB models, several small but significant differences between the systems, as well as

preferences between laboratories, have been introduced (Garberg 1998; de Boer and Sutanto 1997), an ongoing process as shown by recent papers (Thomsen et al. 2017; Veszelka et al. 2018; Stone et al. 2019). In the following sections, current in vitro models of the BBB are briefly surveyed; for greater detail on specific systems, the reader is referred to the corresponding key publications.

8.2.1 Isolated Brain Capillaries

Brain capillaries can be isolated from animal as well as human autopsy brains using mechanical and/or enzymatic procedures (Pardridge 1998; Miller et al. 2000). Typically, the capillary fragments consist of endothelial cells ensheathed by a basement membrane containing pericytes to which remnants of astrocytic foot processes and nerve endings may cling. Often preparations contain small venules and precapillary arterioles and hence smooth muscle cells. Isolated brain capillaries are metabolically active, although a significant loss of ATP and hence activity during the isolation procedure has been reported (Pardridge 1998). As the luminal surface of isolated brain microvessels cannot easily be accessed in vitro, most studies investigate the abluminal properties and function of the BBB. The technique has been used with porcine, rat, and mouse microvessels and has given detailed insights into the cellular and molecular mechanisms regulating transport at the BBB and blood-spinal cord barrier, especially for P-glycoprotein (Pgp) (Miller 2010; Campos et al. 2012).

After isolation, brain microvessels can be stored frozen at -70°C, thereby providing a versatile tool for several applications and a viable source for the cultivation of brain microvessel endothelial cells (Audus et al. 1998). In earlier studies, isolated brain capillaries were used to examine receptor- and adsorptive-mediated endocytosis and solute transporter systems (Pardridge 1998; Fricker 2002). Confocal and live imaging microscopy has expanded possible studies, for example, transendothelial transport of fluorescent substrates for drug transporters (Miller et al. 2000) (Fig. 8.4) and regulation of MRP, BCRP, and PgP function in human, porcine, and rodent capillaries by glutamate (Bauer et al. 2008; Salvamoser et al. 2015; Luna-Munguia et al. 2015).

Isolated capillaries have also proven a valuable resource to characterize BBB mRNA and key transport protein expression, comparing different species and luminal vs. abluminal polarization (Shawahna et al. 2011, Ito et al. 2011a, Uchida et al. 2011b; Hoshi et al. 2013; Kubo et al. 2015) (see Sect. 8.2.6.2). Isolated brain capillaries from both animals and human with a neurological disorders or genetic alteration are contributing to elucidation of the role of the BBB in CNS pathophysiology (Wang et al. 2012; Hartz et al. 2012).

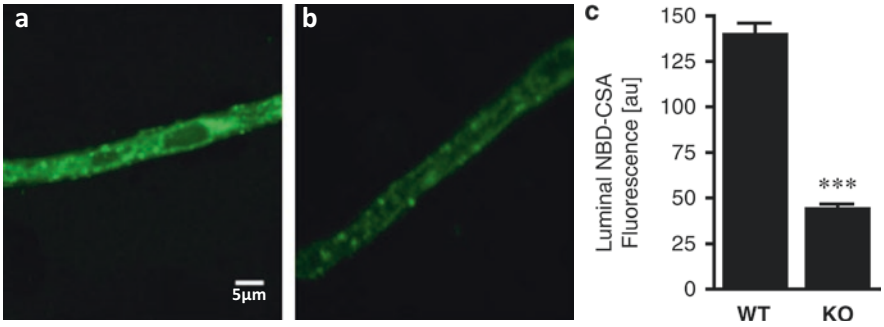

Fig. 8.4 Isolated mouse brain capillaries to study P-glycoprotein function. P-glycoprotein transport function measured as luminal accumulation of fluorescent Pgp-specific substrate NBD-CS. NBD-cyclosporin A in isolated brain capillaries from (**a**) wild-type and (**b**) CF-1 Pgp-deficient mice. (**c**) Image analysis. Methods: Brain capillaries were isolated from wild-type (CF1TM) and CF-1 P-glycoprotein-deficient mice (KO; CF1-Abcblamds). P-glycoprotein transport activity was determined by exposing capillaries to 2 fM NBD-CSA for 1 h and measuring luminal fluorescence using confocal microscopy and image analysis. (Data are mean ± SEM for 7 capillaries for each preparation of 20 mice; shown are arbitrary units (0–255). Statistics: *** P<0.001 (Student t-test). Hartz AMS and Bauer B, unpublished data, with permission)

8.2.2 Primary and Low Passage Brain Endothelial Cells

Apart from isolated brain microvessels, the system next closest to in vivo is primary BECs which are isolated from or grow out of brain capillary fragment: Primary as well as low passage BECs retain many of the endothelial and BBB-specific characteristics of the BBB in vivo; however, these features are often downregulated or even lost with increasing passage if not re-induced. The most successful way to retain BBB features is through co-culture with inducing cells such as astrocytes, pericytes or neurons either in noncontact formation (Fig. 8.1b) or in contact formation (Fig. 8.1c). In addition, most protocols modify the culture medium once cells reach confluence, withdrawing serum to reduce proliferation and encourage cell-cell contact, including cAMP to encourage basement membrane formation and glucocorticoids to improve tight junction protein expression (Hoheisel et al. 1998; Thomsen et al. 2017).

Rat and Mouse Models Due to the much higher yield of BECs from bovine and porcine brains compared to rat brains (up to 200 million cells per porcine brain, compared to 1–2 million cells per rat brain), the former species currently represent the most popular source for in vitro BBB models both in academia and industry. However, primary cultured rat and mouse systems continue to be useful for investigation of pharmacology and transport, in studies where specific antibodies for larger species are lacking, and for comparison with standard in vivo rodent (rat, mouse) models used for PKPD analysis. The increasing availability of high-quality BECs from commercial sources has also added to the consistency and continued use of these models.

The use of primary rodent models for transendothelial permeability measurements was until recently limited by the relatively leaky monolayers generated (TEER 150–200 Ω.cm^2 due to the small flaws caused by contaminating pericytes, which are less of a problem in the bovine and porcine systems) (Patabendige et al. 2013a, b). However, Watson et al. (2013) showed that improvements in methods through generation of purer rat primary cultures, co-culture with mixed glia from the same species ("syngenic" culture), and short trypsinization times can give higher TEER of up to 600 Ω.cm^2. Inclusion of puromycin to eliminate contaminating pericytes from the monolayer is a relatively simpler procedure to generate consistent monolayers with suitable TEER ~200 Ω.cm^2 and low paracellular permeability $P_e \sim 3 \times 10^{-6}$ cm/s (Stone et al. 2019).

Recent studies with primary rat and mouse BECs have focused on in vitro pathological models to mirror in vivo rodent studies, for example, the effects of stroke (Venkat et al. 2021; Kong et al. 2021), inflammation and T cell migration (Hamminger et al. 2021), and demyelination syndrome (Scalisi et al. 2021).

Bovine Models Bovine BEC cultures are widely used, but differences between the procedures have developed historically in different BBB groups. Pioneered by Bowman et al. (1983) and later modified by Audus and Borchardt (1986) in the USA, bovine BECs are typically isolated by a combination of mechanical and enzymatic protocols and originally grown in monoculture (Miller et al. 1992) with early studies showing TEER in the range 160–200 Ω.cm^2 and sucrose permeability $10 – 20 \times 10^{-6}$ cm/s (Raub et al. 1992; Shah et al. 2012).

In Europe, several modifications to the protocol have greatly enhanced the model's BBB properties. The group of Cecchelli and coworkers (Dehouck et al. 1990; Cecchelli et al. 1999) pioneered the omission of enzymatic steps in the bovine BEC isolation, using instead micro-trypsinization and subculturing of endothelial cell islands (clones) that grow out of brain capillaries selectively attached to a defined extracellular matrix. The most recent protocols use BECs after a single passage, supplemented with dexamethasone and cAMP plus phosphodiesterase inhibitor (Eigenmann et al. 2016; Kristensen et al. 2020; Goldeman et al. 2020). BECs can reach TEERs of 600 Ω.cm^2 in monoculture, increasing to 1000–2000 Ω.cm^2 in contact co-culture with rat astrocytes (Fig. 8.1c). Co-culture also aids in reducing paracellular permeability (mannitol $P_{app} < 1 \times 10^{-6}$ cm/s; Tornabene et al. 2019) and in halting or counteracting the loss of specific BBB markers (Goldeman et al. 2020).

The model has been successfully used to study BBB transport (e.g., Wallace et al. 2011) and rank-order compounds according to their BBB permeability (Lundquist et al. 2002; Eigenmann et al. 2016); higher throughput variants of the model have been introduced for drug screening and toxicity testing (Culot et al. 2008; Vandenhaute et al. 2012), and it is one of the few models which have proven suitable for the study of receptor-mediated transcytosis (Candela et al. 2010).

Porcine Models Galla and coworkers (Hoheisel et al. 1998; Franke et al. 1999, 2000) developed a model based on porcine BECs (PBEC model) cultured without serum or astrocytic factors but in the presence of the tight junction protein

differentiating agent hydrocortisone. In their hands, this model gives among the highest TEER values measured in vitro thus far (400–1500 Ω.cm^2 with VO monitoring, or higher in IS systems, with sucrose permeability down to $1^{-4} \times 10^{-6}$ cm/s). The model has been used as a screening tool for CNS penetration of small drugs (Lohmann et al. 2002) and nanocarriers (Qiao et al. 2012) and for a number of mechanistic studies of BBB transporters and cell-cell interaction in the neurovascular unit (NVU). Using this model, Cohen-Kashi Malina et al. (2009, 2012) showed an increased TEER of the PBECs, from 415 Ω.cm^2 in monoculture to 1112 Ω.cm^2 in contact co-culture (Fig. 8.1c). The model was sufficiently tight and polarized to examine the role of endothelial and glial cells in glutamate transport from the brain to blood (Cohen-Kashi Malina et al. 2012).

A different PBEC method originally developed by Louise Morgan and the group of Rubin (Eisai Laboratories, London), based on a method for bovine BECs (Rubin et al. 1991), was reintroduced by Skinner et al. (2009) using serum-free medium and supplements hydrocortisone and cAMP plus phosphodiesterase inhibitor. Further optimization including a growth phase with plasma-derived serum rather than fetal serum and noncontact co-culture with rat astrocytes (Patabendige et al. 2013a, b; Nielsen et al. 2017) gave maximum TEER of 2400 Ω.cm^2; permeability to LY was $<1 \times 10^{-6}$ cm sec^{-1} at average TEER 1249 Ω.cm^2 (Nielsen et al. 2017). The P_{app} is uniformly low in BECs with TEER >500 Ω.cm^2, so for this model a threshold is set to 500 Ω.cm^2 to be used for experiments. Interestingly, co-culture with porcine pericytes reduced TEER compared to culture with porcine astrocytes (Thomsen et al. 2015), which underlines the complexity of cell-cell interactions.

The model shows good functional and polarized expression of transport proteins (Patabendige et al. 2013a; Kubo et al. 2015), tight junctions, enzymes, and receptors (see Nielsen et al. 2017). The model has been used to study receptor-mediated transcytosis (RMT) for interleukin-1 (Skinner et al. 2009) and LRP-1 and RAGE substrates (Wainwright et al. 2020) and more recently for studies of nanoparticle delivery of monoclonal antibodies to the brain (Woods et al. 2020) and effect of inhibition of Pgp, MRP5, and BCRP on amyloid clearance from brain to blood (Shubbar and Penny 2020).

Human Models The limited availability of human brain tissue makes primary human BECs a precious tool for the study of the human BBB at the cellular and molecular level (Dorovini-Zis et al. 1991). The source material usually derives either from autopsies or biopsies (e.g., temporal lobectomy of epilepsy patients), and the most popular applications are studies related to the BBB in CNS diseases. Commercial human brain endothelial cells are increasingly available, although batch-batch variation may pose problems. Human BEC monolayers are fragile in culture, contributing to low TEER values of 120–180 Ω.cm^2 (Mukhtar and Pomerantz 2000; Giri et al. 2002). Co-culture with combinations of NVU cells including pericytes and neurons does not necessarily increase TEER but does speed up response to dexamethasone supplement and increases sensitivity to oxygen-glucose deprivation (Stone et al. 2019).

A great advantage of primary human models is the ability to generate cultures from tissue originating from patient pathology samples (Giri et al. 2002) and to mimic pathology and interrogate cell signaling in a human model including SARS-CoV-2 infection (Larochelle et al. 2012; Liu and Dorovini-Zis 2012; Sugimoto et al. 2020; Nascimento Conde et al. 2020). In addition, these models have also been used to study drug transport (Riganti et al. 2013) and nanoparticle permeation (Gil et al. 2012).

8.2.3 Immortalized Brain Endothelial Cell Lines

Primary cultured BECs have been successfully used as in vitro model of the BBB; however, their widespread and routine use has been restricted mainly by the time-consuming and often difficult preparation of the system which limits the continuous and homogeneous supply of biological assay material. Therefore, attempts have been made by several laboratories to immortalize primary BECs, thereby avoiding the lengthy process of cell isolation.

The first generation of immortalized CNS barrier cell lines (first publication 1988–2000) involved introducing genes such as polyomavirus T antigen (bEND.3 cells), adenovirus ETA gene (RBE4), or SV40 large T antigen (many) (Table 8.2). Subsequently, conditionally immortalized cell lines have been established by using transgenic mice and rats harboring the temperature-sensitive SV40 large T antigen gene (tsA58 T antigen gene) (Terasaki and Hosoya 2001; Terasaki et al. 2003). The advantage is that only small amounts of tissue are needed to establish a cell line, and the cell lines generated show better maintenance of in vivo functions proliferate well and reach confluence in 3–5 days. The gene is stably expressed in all tissues, and cell cultures can easily be immortalized by activating the gene at 33 °C (Ribeiro et al. 2010). The technique has been used to generate both brain endothelial and choroid plexus cell lines.

Of immortalized brain endothelial cell lines introduced in 1988–2000, several have proven reliable and popular and are still in use (Table 8.2). The models have been characterized to varying degrees, but all shared a common weakness, i.e., insufficient tightness when grown as a cell monolayer on a porous membrane. Innovations to improve tightness have focused on the same interventions used for primary cells: co-culture with inducing cells and addition of glucocorticoids such as hydrocortisone or dexamethasone (see Sect. 8.2.2). The situation more recently has significantly improved, as detailed further below, and the most recent addition to BBB models, human stem cell-derived endothelial-like cells, has enormous promise to combine human cells with a tight monolayer and stability through multiple passages.

Bovine and Porcine Cell Lines As good primary cultured bovine and porcine BECs are now routinely produced in several groups, the use of immortalized bovine

and porcine models showing more restricted features (Reichel et al. 2003) is less widespread.

Rat and Mouse Cell Lines One of the first, and still most widely used, immortalized in vitro models is the mouse bEND.3 cell line derived originally from BALB/c mouse brain endothelia infected with the polyomavirus middle T oncogene (Williams et al. 1989; Montesano et al. 1990). The ease of availability and use, consistent generation of monolayers, and ability to compare with mouse WT and KO in vivo studies make this a popular choice. The bEND.3 cell line expresses the relevant tight junctions and transport proteins but does not generate high TEER, possibly because of inherent proteolytic activity (Montesano et al. 1990). TEER is typically 40–50 $\Omega.cm^2$ in monoculture, increasing to 70–80 $\Omega.cm^2$ in co-culture with astrocytes, and permeability to LY or sodium fluorescein ranges from 3 to 15×10^{-6} cm/s (Seok et al. 2013; Martins et al. 2016; García-Salvador et al. 2020). Attempts to improve culture systems using puromycin, for example, have not yielded success (Puscas et al. 2019).

Most recently, bEND.3 cells have been used for the study of brain delivery of large molecules or nanoparticles (Zhang et al. 2021; Wainwright et al. 2020), drug screening in comparison with in vivo mouse data (Puscas et al. 2019), and stroke models (Baumann et al. 2021).

In an interesting breakthrough, Förster et al. (2005) returned to the earlier cell transduction technology used for bEND3 and bEND5 to generate mouse cEND cell: which uniquely among immortalized brain endothelial cell lines can produce tight monolayers, with reported TEER up to >800 $\Omega.cm^2$. The details of the immortalization method have been published, and the cells have been used for studies on the involvement of glucocorticoids on tight junction regulation and on hypoxia and multiple sclerosis (Burek et al. 2012).

For rat, the RBE4 and GP8/GPNT cell lines are still in use, although less frequent in the last 2 years (Table 8.2), and have proven useful for a broad array of topics ranging from mechanistic transport studies to receptor-mediated modulation and inflammatory responses. Many of the currently available immortalized rat and mouse cell lines, especially conditionally immortalized lines, have been generated in Japan and are widely used, often in parallel in vivo/in vitro studies, especially for identification and examination of carrier-mediated transport (Ito et al. 2011b, c; Lee et al. 2012; Tega et al. 2013).

Human Cell Lines Immortalization of human BECs has proven much more difficult than for BECs of other species, but several human cell line models are reported (Reichel et al. 2003; Deli et al. 2005) suitable for examination of the physiology, pharmacology, and pathology of the human BBB in vitro and as a screening tool for CNS penetration.

The most widely used is the hCMEC/D3 cell line (Table 8.2) introduced by Weksler et al. (2005, 2013), building on the author's prior experience developing rat RBE4, GP8.3, and GPNT cell lines. hCMEC/D3 cells are contact-inhibited, can

reach confluence in as quickly as 48 h, and retain features for up to ~30 passages (Weksler et al. 2005; Schrade et al. 2012) making them a robust laboratory tool. Like most cell line models, TEER is typically low, around 35–50 Ω.cm^2, however, modifications including astrocyte co-culture, addition of simvastatin, hydrocortisone, or lithium activating the Wnt system, can elevate TEER to 90–200 Ω.cm^2 with permeability to LY or sodium fluorescein between 10 and 20 × 10^{-6} cm/s (Förster et al. 2008; Schrade et al. 2012; Veszelka et al. 2018; Gericke et al. 2020; García-Salvador et al. 2020).

hCMEC/D3 has rapidly been adopted as an immortalized model of choice for studies where TEER is not a major issue, e.g., macromolecule and nanoparticle uptake and transport (Markoutsa et al. 2011; Yamaguchi et al. 2020), pathology, and cell signaling (Ito et al. 2017; Alam et al. 2020). A review by Weksler et al. (2013) summarizes many of the useful applications of the model and gives a balanced view of its strengths and weaknesses.

Human Stem Cell Derived Developing a stable, human BBB model is essential to fully investigate CNS drug delivery and pathophysiological targets that are translatable to patients. Since primary and immortalized human BECs have limitations as discussed above, efforts have been made to develop a suitable BBB model using human stem cells. These have the advantage of a human genotype and so the added potential for generating cells from patients to study diseases (Raut et al. 2021) or personalized drug interactions. A disadvantage is the cells do not originate from brain endothelium, but rather they are pluripotent or hematopoietic stem cells in origin, which must be differentiated and induced to express BBB features and suppress non-BBB features.

The induced pluripotent stem cell model (iPSC) was developed by the Shusta group involving initial differentiation of stem cells into endothelial cells and co-culture with neural cells providing Wnt/β-catenin signaling and then purification and further maturation of the endothelial cells to develop a full BBB-like phenotype (Lippmann et al. 2012, 2013, 2014). Multiple laboratories are applying these methods, and the cells reliably generate TEERs of 1000–2000 Ω.cm^2 with the highest reported over 5000 Ω.cm^2 and low paracellular permeability of 0.5–2 × 10^{-6} cm/s (Lippmann et al. 2014; Le Roux et al. 2019; Raut et al. 2021).

Brain-like endothelial cells (BLEC) derive from CD34+ hematopoietic stem cells isolated from cord blood which makes them relatively easily harvested and available (Cecchelli, et al. 2014). Cells are differentiated and then co-cultured with pericytes to induce BBB features. The TEERs are superior to immortalized or primary human BBB models but are variable between laboratories ranging from 40 to 360 Ω.cm^2 with permeability of 5–15 × 10^{-6} cm/s (to LY or sodium fluorescein; Rand et al. 2021; Santa-Maria et al. 2021). Applying shear stress using a flow system (see Sect. 8.2.4) improves TEER to >400 Ω.cm^2 (Santa-Maria et al. 2021), making these cells suitable for the more complex methods described below.

There has been rapid progress on signaling and transcription factors to differentiate stem cells into a mature BBB phenotype (Lu et al. 2021; Roudnicky et al. 2020a), and as a consequence, this may benefit other BBB models. For example, factors

identified that increase expression of claudin-5 in hPSCs were applied to primary human cells to improve BBB phenotype (Roudnicky et al. 2020b); changes in gene expression following pericyte co-culture with BLECs have identified molecular processes in BBB formation (Heymans et al. 2020).

An area for future work is to characterize these models, looking for "non-BBB" features and ensuring they are downregulated so that erroneous interpretations about BBB function are not made. For example, the iPSC model expresses some epithelial adhesion proteins and transporters, but these can be downregulated with endothelial transcription factors (Lu et al. 2021). The reproducibility and transferability of these models will also be critical features in the future, but these models show enormous promise in taking the field forward.

Non-BBB Cell Lines It is generally difficult to make BEC cell lines switch from the exponential growth phase after cell seeding to a more static phase of cell differentiation after the cells have reached confluence. Therefore, most immortalized cell lines are less applicable for studies requiring a tight and stable in vitro barrier, but they have proven useful for mechanistic and biochemical studies requiring large amounts of biological material as described above. However, the insufficient tightness of immortalized BEC lines renders them unsuitable for use in simple BBB permeability screens. Therefore, some groups have turned to other cell lines which, although of non-brain origin, either express sufficient brain endothelial features for functional and permeation studies such as ECV304/C6 (Hurst and Fritz, 1996; Neuhaus et al. 2009; Wang et al. 2011) or prove on validation to be useful predictors of passive and Pgp-mediated CNS penetrability of compounds, such as MDCK cells engineered to overexpress human Pgp (MDCK-MDR1) and Caco-2 cells (Summerfield et al. 2007; Hellinger et al. 2012).

8.2.4 Complex BBB Models: 3D Models, Dynamic Flow, and Microfluidics

It would be expected for in vitro models retaining complex features of the in vivo NVU that they would be more successful in showing a functional BBB phenotype. In cell culture models, the inclusion of pericytes can be beneficial, depending on the differentiation state of the pericytes (Thanabalasundaram et al. 2011). Not all in vitro models are reported to respond positively to pericytes (co-culture does not improve TEERs in primary human or porcine models; Stone et al. 2019; Thomsen et al. 2015), but many examples of barrier-inducing and stabilizing effects of pericytes on BBB function have been demonstrated (Fig. 8.5) (e.g., Nakagawa et al. 2009; Vandenhaute et al. 2011), and a practical commercial rat tri-culture model is available. A more complex model development is the "spheroid" or brain organoid, which is a sphere of endothelial cells surrounding a monolayer of pericytes and astrocyte core. These 3D cell systems spontaneously self-organize in a hanging droplet culture plate or in a well with ultralow attachment (Urich et al. 2013;

Kumarasamy and Sosnik 2021) and have been used to study nanoparticle uptake, for example. While these recapitulate the cell-cell interactions, it is difficult to determine detailed BBB function.

Another example of more closely mimicking the in vivo environment is growing BECs in porous tubes with luminal flow and external astrocytes to aid barrier induction (Stanness et al. 1996, 1997; Janigro et al. 1999). This "dynamic in vitro" (DIV) BBB model (Fig. 8.6) proved an important innovation and convincingly demonstrates not only improved junctional tightness but also other BBB features reflecting the differentiating effects of flow. There is growing interest in combining 3D, triculture, and flow in a single miniaturized "microfluidic" platform capable of mimicking more closely the in vivo conditions, but with less cell volume and need for reagents. Pioneering studies established the feasibility of the method and scope for miniaturization (Booth and Kim 2012; Griep et al. 2013; Prabhakarpandian et al. 2013), with BBB cell line models RBE4, bEND3, and hCMEC/D3. The positive effects of flow in DIV and microfluidic systems can be demonstrated in primary cells, immortalized cells, and more recently stem cell-derived models, for example, TEER is improved up to 500 Ω.cm^2 in primary human cells and BLEC (Cucullo et al. 2011; Santa-Maria et al. 2021), 1000 Ω.cm^2 in hCMEC/D3 (Partyka et al. 2017), and 4000 Ω.cm^2 in iPSCs (Grifno et al. 2019). However, the complexity of the geometry (multiple hollow fibers) in this model and the assumptions made in calculating TEER from the current measured make it difficult to compare TEER values with those from flat filter configurations.

Despite the undoubted improvement in BBB characteristics with these systems, these models are more difficult to set up and maintain than standard mono- or co-cultured models (Fig. 8.1) and have not yet been fully assessed for the whole range of BBB features including vesicular transport (Naik and Cucullo 2012; Abbott 2013). There is also wide variation between groups in the BEC cells used and the species and types of co-cultured cells; a recent review by Bhalerao et al. (2020) gives an excellent overview of the challenges in comparing between groups. Many questions could be addressed in such systems, including the contribution of differential flow rates/shear stress to the observed heterogeneity of endothelial cytoarchitecture and function in different segments of the vasculature (Ge et al. 2005;

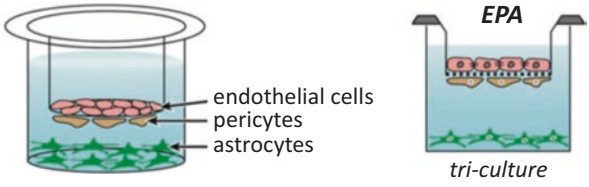

Fig. 8.5 Configuration for tricellular BBB co-culture model, reflecting the organization of the neurovascular unit (NVU). As for Fig. 8.1b, but here with addition of pericytes. Endothelial cells E on the top of the filter, pericytes P on the underside of the filter, and astrocytes A in the base of the well (EPA arrangement). (Redrawn by R Thorne, based on Nakagawa et al. 2009, with permission)

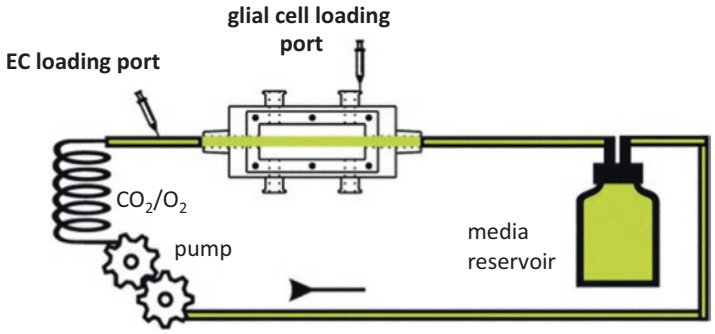

Fig. 8.6 Dynamic in vitro BBB model, DIV-BBB. Diagram showing cartridge containing replaceable bundle of hollow porous polypropylene fibers (capillary tubes) (yellow) suspended in the chamber and in continuity with a medium source through a flow path consisting of gas-permeable silicon tubing. A servo-controlled variable-speed pulsatile pump generates flow from the medium source through the capillary tube bundle and back. The circulatory pathway feeds both endothelial cells (EC) growing on the luminal surface of the capillary tubes and glia growing abluminally on their outer walls. The model has been used to assess the effects of flow on endothelial physiology, pathophysiology, and leukocyte trafficking. (From Cucullo et al. 2002, with permission)

Macdonald et al. 2010; Saubaméa et al. 2012; Paul et al. 2013; *cf* Ballermann et al. 1998). Given the complexity of the microfluidics chambers, these are not likely to be suitable for high-throughput permeability assays at least in the short term, but meanwhile the generation of detailed mechanistic information is likely to be the most valuable output. An important advantage will be the ability to test barrier cells from different species and with different pathologies, under equivalent conditions.

8.2.5 Application of In Vitro Models for BBB Drug Permeability Assay

A realistic in vitro assay system for screening and optimizing NCEs should combine as many features as possible of the in vitro BBB yet be suitable for medium to-high-throughput screening. Most pharmaceutical/biotech companies already have screens for intestinal permeability (generally Caco-2) and, for "Pgp-liability," often MDCK-MDR1 cells (Summerfield et al. 2007), so a convenient and pragmatic system is to expect early-stage screening on such models and later refinement in a more "brain-like" system. A possible "screening cascade" involving early in silico modeling, then non-brain epithelial models, and finally CNS barrier models may be practical (Abbott 2004). However, given the very different morphologies of endothelial cells and the epithelial cells Caco-2 and MDCK, especially in cell thickness, luminal membrane microstructure, glycocalyx composition, junctional structure, and organelle content (Fig. 8.7) together with physiological differences in transcytosis mechanisms, transporter, and enzyme function, caution still needs to be applied in such a sequential screen (see also Lohmann et al. 2002).

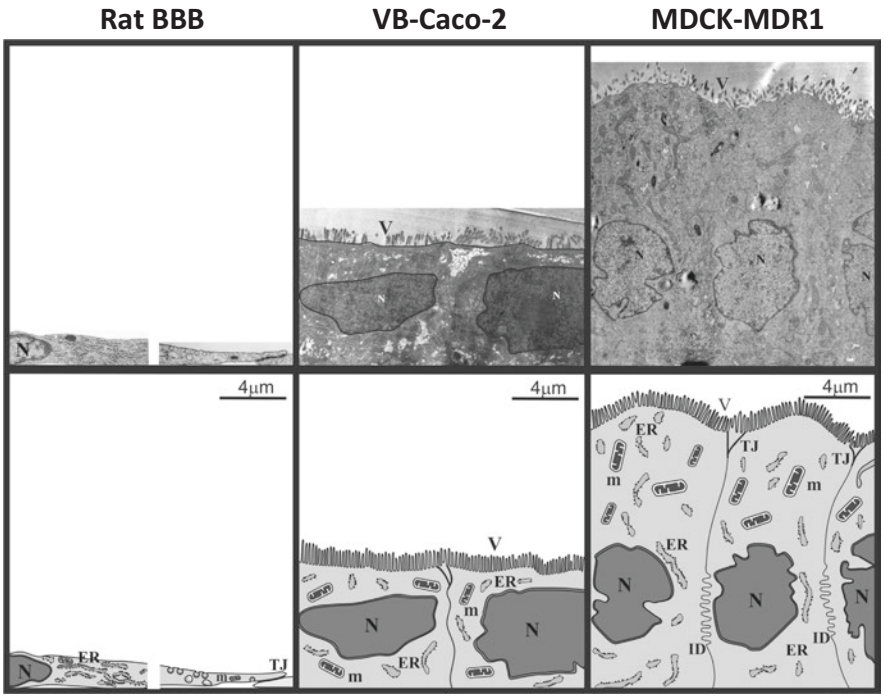

Fig. 8.7 Electron micrographs of cell cultured rat brain endothelium, VB-Caco-2 and MDCE-MDR1 cell cytoarchitecture, with drawings below. VB-Caco-2 cells were created by growing Caco-2 cells in 10 nM vinblastine (VB, Pgp substrate) for at least six passages to elevate P-g expression. (*ER* endoplasmic reticulum, *ID* interdigitations, *m* mitochondrion, *N* nucleus, *TJ* intercellular tight junctions, *V* microvilli. From Hellinger et al. 2012, with permission)

Most studies for CNS-specific permeability screening have focused on the BBB as the largest surface area blood-CNS interface, closest to neurons, but there is growing awareness of the need for assay systems of the choroid plexus reflecting especially the transport and enzymatic importance of this barrier (Strazielle and Ghersi-Egea 2013). A medium- to high-throughput BBB system using bovine endothelial cells exposed to glial-conditioned medium is available (Culot et al. 2008), and primary cultured porcine cells are also suitable either as monocultures or co-cultures with astrocytes (Patabendige et al. 2013a). Hellinger et al. (2012) compared a rat tri-culture model (TEER ~200 $\Omega.cm^2$) with Caco-2 and MDCK-MDR1 cells in screening ten compounds (selected for predominantly passive permeation, efflux transport, or both) and concluded that for passive permeability and Pgp-liability, the epithelial layers gave better resolution, while the BBB model would have advantage in reflecting other in vivo BBB transporters. However, with a more limited drug set, Mabondzo et al. (2010) using human primary BECs concluded they were better than Caco-2 cells at correlating with in vivo human PET ligand uptake (detailed below), which may reflect important differences in species, drug set, or the culture protocols of the in vitro systems used.

8.2.6 *In Vitro-In Vivo Correlations (IVIVC)*

Since the earliest in vitro BBB permeability assays (e.g., Dehouck et al. 1990; Cecchelli et al. 1999), there has been interest in comparing the performance of the in vitro models against permeability data generated in vivo, typically by constructing an in vitro vs. rodent in vivo permeability plots and determining the correlation (in vitro-in vivo correlation, IVIVC). Rodent in vivo data used have been either measurements of Brain Uptake Index (BUI) or permeability data derived from in situ brain perfusion, the K_{in} (unidirectional influx coefficient), or the derived P_c (transcellular permeability). However, the relatively leaky tight junctions in vitro (high paracellular permeability) and the presence of unstirred water layers (or aqueous boundary layers, ABL; Youdim et al. 2003) weaken the correlation (Avdeef 2011).

Despite these limitations, reasonable correlations can be generated, especially for primary cells. For example, IVIVC using primary mouse BECs vs. in vivo mouse brain-to-blood ratio gave better correlation than bEnd.3 cell line vs. in vivo ($r^2 = 0.765$ primary cells; $r^2 = 0.019$ bEND.3; Puscas et al. 2019). The increasing availability of agents suitable for human positron emission tomography (PET) imaging now allows comparison of in vitro human BBB models with human brain uptake. For example, Mabondzo et al. (2010) compared transport of seven drugs across primary human BECs co-cultured with syngenic astrocytes to human brain PET-MRI data and showed excellent correlation ($r^2 = 0.90$) and being better than Caco-2 vs. human brain PET ($r^2 = 0.17$). Le Roux et al. (2019) similarly showed good human IVIVC correlation for eight PET ligands, using iPSC-derived BECs ($r^2 = 0.83$). It will clearly be important to extend these studies to a wider drug library and compare other in vitro BBB models to human PET data.

8.2.6.1 Unstirred Water Layer, Paracellular Permeability, and Intrinsic Permeability Calculation

Building on quantitative biophysical models validated in epithelia and applying his software *p*CEL-X, Avdeef (2011) used literature values (to 2008) of permeability from several different in vitro BBB and epithelial models and deconvoluted the apparent permeability P_e of the endothelial barrier into its three components: P_{ABL}, P_C, and P_{para}, (ABL, transcellular and paracellular permeabilities, respectively). Finally, P_0, the intrinsic (charge-corrected) permeability, was calculated from P_C by incorporating the pK_a value(s) of the molecule. Figure. 8.8 shows the log-log IVIVC of P_0 data from monocultured porcine brain endothelium vs. P_0 data from rodent in situ brain perfusion studies. The correlation coefficient r^2 for the IVIVC (0.58) was greater than that for the uncorrected in vitro data, P_e vs. P_C in situ (0.33). The porcine BBB model also performed better than bovine, rodent, and human models in this study. By applying the method to permeability data from the tightest current

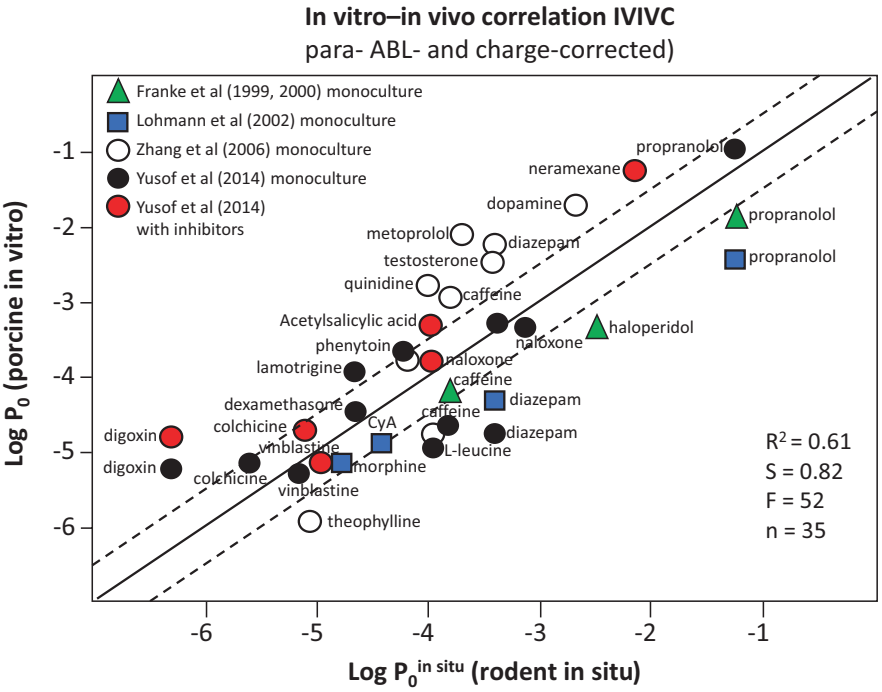

Fig. 8.8 In vitro-in vivo correlation analysis (IVIVC). Intrinsic transcellular permeability (P_0) data were compared with in situ brain perfusion data from rodent. P_{app} data were corrected for aqueous boundary layer (ABL) permeability, paracellular permeability, filter restriction, and possible uptake of the charged species. In situ brain perfusion data from rodent were collected from the literature and analyzed using the pKa FLUX method to derive P_0 (Dagenais et al. 2009; Suzuki et al. 2010; Avdeef 2011, 2012). The predictions for in situ BBB permeation of acetylsalicylic acid and neramexane (calculated from pCEL-X) and dexamethasone and metoprolol (Caco-2 values) were used in the analysis (underlined). The solid line is the linear regression with r^2 value of 0.61. The dashed line is the reference "line of identity." (Modified from Yusof et al. 2014 with permission)

in vitro BBB models, the correlations are expected to improve. The method helps to identify the most reliable in vitro models for predicting in vivo permeability and to correct the data obtained from leakier models.

8.2.6.2 Transcriptomics, Proteomics, and PKPD Modeling

Transcriptome examination and quantitative proteomics of freshly isolated brain capillaries and purified brain endothelial cells have helped determine the degree to which in vitro models reflect the in vivo condition and how closely models from other species resemble the phenotype of the human CNS barriers (Kamiie et al. 2008; Daneman et al. 2010a; Ohtsuki et al. 2011; Hoshi et al. 2013; Al Feteisi et al. 2018; Chaves et al. 2020). These techniques are also revealing changes in BEC

protein and mRNA expression related to disease models such as stroke and seizures (Tornabene et al. 2019; Munji et al. 2019; Gerhartl et al. 2020) and shedding light on the role of miRNAs (Kalari et al. 2016). In the future, it should be possible to combine information from in vivo and in vitro studies (Ito et al. 2011b, c) with quantitative proteomics (Uchida et al. 2011a, b, 2013; Kubo et al. 2015) to generate data for PKPD and "physiologically based pharmacokinetic" (PBPK) modeling and for prediction of human CNS free drug concentrations (Shawahna et al. 2013), based on data including information generated in in vitro models from different species (Ball et al. 2012). The ultimate aim will be to permit reliable in vitro-in vivo extrapolation (IVIVE) to human brain (Ball et al. 2013). BBB-specific transcriptome databases, such as the BBBomics hub http://bioinformaticstools.mayo.edu/bbbomics/ (Kalari et al. 2016) and the European Brain Barriers Training Network BBBhub http://bbbhub.unibe.ch/ (Heymans et al. 2020), will be valuable resources in this endeavor.

8.2.7 How to Select an Appropriate In Vitro BBB Model

It is clear that a wide range of models are available for studies of the BBB relevant to normal physiology and pathological situations and to test and optimize CNS delivery of appropriate therapies. Careful selection with a variety of controls in place can give valuable information about the role of the BBB in pathology and the rate and extent of entry of therapeutics into the CNS. These models are helping to refine a variety of formulations and constructs to improve their value in a range of diseases.

For scientists starting a new BBB project without prior experience, collaboration with an established group or groups is recommended, including adopting their well-characterized cell or cell line models if these are suitable for the application planned (Table 8.3).

8.2.8 Epithelial CNS Barriers

8.2.8.1 Choroid Plexus Epithelial (CPE) Cells

The choroid plexus is relatively straightforward to isolate with cell viability maintained for several hours, permitting studies of uptake and efflux, but without defined polarity (Gibbs and Thomas 2002). When polarity of transport is important, perfusion and isolation of sheep choroid plexus permits studies of vectorial transport across the epithelium (Preston et al. 1989). Primary culture models of rodent, porcine, and human CPE have been developed (see Baehr et al. 2006), but the most readily available human material is from fetal material or CP papilloma, which may not accurately reflect normal function (Redzic 2013). Resistances of 100–600 $\Omega.cm^2$ have been

Table 8.3 How to select an appropriate in vitro BBB model (see text)

Property of interest	Recommended cell model(s)	Check
Transendothelial permeability of small compounds (<500 MW), detecting both passive and transporter-mediated flux	Primary cultured cells: —Without astrocytes: porcine —With astrocytes: bovine, porcine —With astrocytes and pericytes: bovine, rat, porcine	Check TEER; aim for high TEER and high dynamic range, giving better discrimination and rank-ordering within a drug series
ABC efflux transporters	Primary cultured system showing in vivo pattern, polarity/localization (bovine, porcine)	Check relative expression compared to human BBB, may permit prediction of PK in human
Transporters mediating brain entry or exit of small compounds via SLCs, ABC transporters	Many models including cell lines show sufficient expression, suitable for uptake and efflux studies	Check expression of transport system of interest; compare with in vivo or primary culture
Metabolic enzymes affecting drug permeation	Many models show sufficient expression	Check model has been characterized for enzymes
Receptor-mediated endocytosis and transcytosis	Primary cultured cells with astrocytes, found critical for full expression and function	Check receptor expression and polarity show features of BBB-type transcytosis rather than "default" non-BBB phenotype
Non-BBB characteristics		Check for absence of epithelial features, e.g., cadherins, transporters, and extensive caveolae

observed (see also Sect. 8.1.3.4), some models are tight enough for demonstration of CSF secretion, and the models have been used for a variety of studies of transport, metabolism, and leukocyte traffic (Redzic 2013; Strazielle and Ghersi-Egea 2013; Monnot and Zheng 2013). A stable continuous subcultivatable porcine cell line PCP-R, (Schroten et al. 2012) and some immortalized cell lines (human Z310, Monnot and Zheng 2013; rat TR-CSFB3, Terasaki and Hosoya 2001) have been introduced. The models have generally not been used for drug permeability screening.

8.2.8.2 Arachnoid Epithelial Cells

It has recently been proven possible to culture arachnoid cells in vitro, which express claudin 1 and generate a TEER of ~160 $\Omega.cm^2$ with restriction of larger solute permeation (Lam et al. 2011, 2012; Janson et al. 2011). Characterization of the expression patterns of drug transporters and enzymes in arachnoid tissue and arachnoid barrier (AB) cells shows expression of both Pgp and breast cancer resistance protein (BCRP); an immortalized cell line of AB cells showed Pgp expression on the apical

(dura-facing) membrane and BCRP on both apical and basal (CSF-facing) membranes (Yasuda et al. 2013). Microarray analysis of mouse and human arachnoid tissue showed expression of many drug transporters and some drug metabolizing enzymes. The consistency across in vitro models and isolated tissue makes it likely that these proteins contribute to the blood-CSF barrier function and confirms that useful in vitro models can be generated and applied to examine these functions in detail.

8.3 Future Directions and Challenges

It is clear that in vitro models will continue to play important roles in generating mechanistic information about cellular and intercellular events in CNS barrier layers, capable of informing a range of applications in health and disease, drug discovery, and drug delivery. Some emerging technologies and their combination offer clear future directions—the challenge will be to make them effective and advance understanding.

We need:

1. Generation of reliable and tight in vitro models of the human BBB, choroid plexus, and arachnoid barriers, reproducing the in vivo condition.
2. Development of an accepted "industry standard" in vitro BBB model, robust, reliable, predictive of human drug PK, and capable of operation in medium- to high-throughput screening of NCEs.
3. Better understanding of TEER measurement in different systems, with accepted calibration protocols, reference thresholds, and intersystem correlations.
4. Better integration of in silico, in vitro, and in vivo models to provide complementary information and more complete characterization of permeability routes and transport systems; we need more projects designed with parallel in vitro and in vivo assessment.
5. More computational modeling with software optimized for CNS barrier models, before, during, and after experiments to better understand and correct for artifacts in permeability-measuring systems.
6. Microfluidics platforms integrating flow, TEER, and other sensors and permitting advanced live cell imaging, suitable for studies of a single barrier cell type or co-cultures reflecting the in vivo condition as within the NVU.

8.4 Conclusions

In the ~40 year history of in vitro CNS barrier models, there have been a number of major advances and of course also many false starts, with natural evolution of the field by which useful, reliable, and informative models become more widely used,

building up the critical mass of basic information from which new developments can take off. Groups developing and adopting in vitro models can learn from the history and current status of the field to ensure that further progress is soundly based and effective and results reliable and applicable between laboratories and across the field. New investigators have available a range of good models and excellent tools and increasingly will work by collaboration to apply them. Exciting times!

8.5 Points for Discussion

1. Imagine a new project in your lab that requires an in vitro model; (a) define the requirements of the model, (b) decide on the most suitable model(s) to use, and justify this choice.
2. Why are leakier BBB models (TEER <200 $\Omega.cm^2$) less suitable for transendothelial permeability screening?
3. For transendothelial permeability measurement, why is it useful to (a) measure the TEER of each filter with cells and (b) make parallel measurements of TEER and permeability of a paracellular marker (P_{app} or P_e), ideally in each experimental run?
4. What are appropriate paracellular markers for the model(s) you selected in (1)?
5. Why has it proven difficult to develop good primary cultured human BBB models?
6. What is an unstirred water layer (aqueous boundary layer, ABL), and why is it a problem for in vitro but not in vivo BBB studies? For transendothelial permeation, which types of compound are most affected by the ABL? If the ABL is not considered, minimized, and corrected for, how would transendothelial permeability measurements be affected?
7. How can in vitro models from different species contribute to prediction of drug PK in human brain interstitial fluid using a process of PBPK?
8. As an exercise, design a microfluidic chamber suitable for studies of transendothelial and transepithelial permeability using CNS barrier cells. What additional features would it provide not generally available for "flat" ("transwell") filter systems? In what ways could these features be important?
9. What are the main differences in generating an in vitro BBB model from human stem cells and from freshly isolated human brain microvessels? How would you select the most "BBB-like" clones from a variety of clones generated from stem cells using different growth conditions and media?

Acknowledgments We are grateful for discussions and comments from many colleagues especially Prof. Maria Deli, Dr. Alex Avdeef, and Prof. Margareta Hammarlund-Udenaes and for redrawing of Fig. 8.1 by Dr. Robert Thorne and for Fig. 8.4 from Dr. Anika Hartz and Dr. Bjoern Bauer.

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