11

THE BLOOD–BRAIN BARRIER AND ITS EFFECT ON ABSORPTION AND DISTRIBUTION

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11.1 INTRODUCTION

The central nervous system (CNS) is a sanctuary site and is protected by various barriers. These regulate brain homeostasis and the transport of endogenous and exogenous compounds by controlling their selective and specific uptake, efflux, and

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metabolism in the brain. Unfortunately, potential drugs for the treatment of most brain diseases are therefore often not able to cross these barriers. As a result, various drug delivery and targeting strategies are currently being developed to enhance the absorption and distribution of drugs into the brain. This chapter discusses the biology and physiology of the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier with respect to drug transport (absorption, distribution), the *in vitro* and *in vivo* methods to measure BBB transport, and the possibilities to deliver large molecular drugs, by viral and receptor-mediated nonviral drug delivery, to the (human) brain.

11.2 BARRIERS IN THE BRAIN

There are three barriers that limit drug transport to the brain parenchyma. These are the blood–brain barrier (BBB) localized in the capillaries in the brain, the blood–cerebrospinal fluid barrier (BCSFB) that is presented by the choroid plexus epithelium in the ventricles, and the ependyma that is an epithelial layer of cells covering the brain tissue in the ventricles and limits the transport of compounds from the CSF to the brain tissue.

11.2.1 Blood–Brain Barrier (BBB)

In 1885 Ehrlich [1] was the first to show evidence for the existence of a barrier between blood and brain. He injected vital dyes intravenously and found that, in contrast to other tissues, it did not stain the brain [1]. His successor, Goldman [2], injected these dyes into the cerebral spinal fluid, after which staining of the brain was observed, but not of the peripheral organs. Since this seminal discovery, much research has been performed on the (patho) physiology and pharmacology of the BBB (see also other reviews [3–9]).

The current knowledge is that the BBB is situated at the interface of blood and brain and its primary function is to maintain the homeostasis of the brain. Furthermore, the BBB is not uniform throughout the brain, since the capillaries in the circumventricular organs (CVOs) are fenestrated [10, 11]. Figure 11.1 gives a schematic representation of the barriers present in the CNS.

The human BBB has a total blood vessel length of approximately 600 km. In fact, every cubic centimeter of the cortex comprises the amazing sum of 1 km of blood vessels. It has an estimated surface area of approximately 20 m^2, which is claimed to be similar to the BCSFB [12, 13]. However, the surface area of the BCSFB is facing the CSF and not the blood, which makes the BBB, based on total blood flow and its wide vascular bed, functionally the most important global influx barrier for solutes to reach the brain [10, 11].

The BBB is mainly formed by brain capillary endothelial cells (BCECs) [14], although other cell types such as pericytes, astrocytes, and neuronal cells also play an important role in the function of the BBB [15–19]. BCECs are different from peripheral endothelial cells, as can be seen schematically in Fig. 11.2 (in which the specific surroundings of the brain capillaries are shown). BCECs have specific characteristics, such as tight junctions, which prevent paracellular transport of small and large (water-soluble) compounds from blood to the brain [14, 20, 21]. Furthermore,

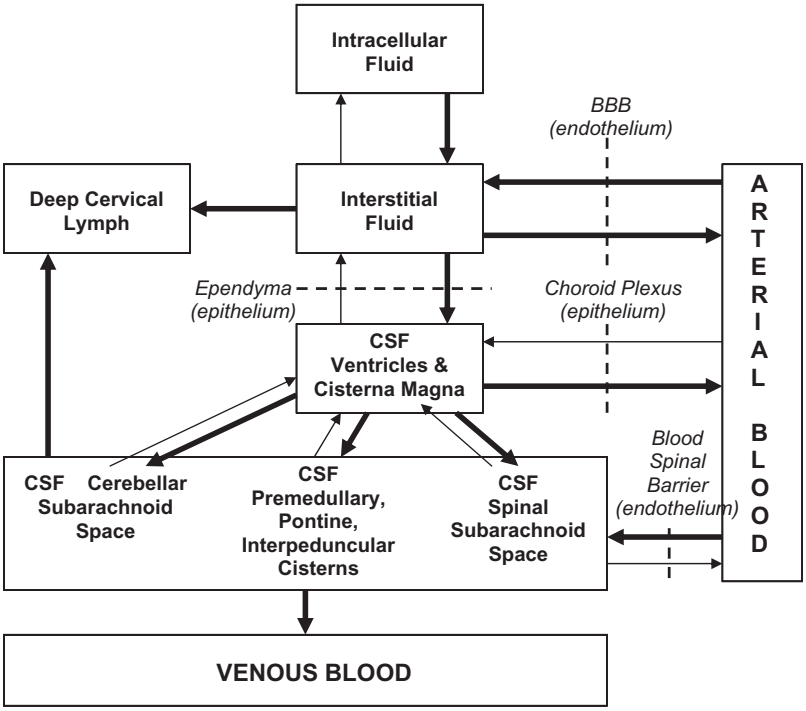


FIGURE 11.1 The various barriers that one can find in the brain (broken lines) representing the blood–brain barrier, the blood–cerebrospinal fluid (CSF) barrier, the brain–CSF barrier, and the blood–spinal barrier. The blood–brain barrier has the largest surface area and is therefore considered to be the most important influx barrier for solutes to reach the brain. In addition, paths are shown for fluid movement (solid arrows) between cerebral intracellular fluid (ICF), intrastitial fluid (ISF), cerebrospinal fluid (CSF), blood, and lymphatics. Thick arrows represent major paths of fluid movement under normal conditions. Thin arrows represent minor paths of fluid movement under normal conditions. (Adapted from Ref. 45.)

transcellular transport from blood to brain is limited as a result of low vesicular transport, high metabolic activity, and a lack of fenestrae [15]. It functions as a physical, a metabolic [22–24], and an immunological barrier [25]. In addition, receptors at the BBB may play a role in brain signaling (e.g., insulin receptor [26], LRP1,2 receptor [27]). These specific characteristics of the BBB are induced and maintained by the (endfeet of) astrocytes, surrounding the BCECs [15, 28], as well as by neuronal endings, which can directly innervate the BCECs [14, 29]. Pericytes also play a role at the BBB, as they share the continuous capillary basement membrane with the BCECs [30]. Their phagocytotic activity forms an additional BBB property [10]. Furthermore, pericytes regulate endothelial homeostasis by inducing the endothelial release of plasminogen-activator inhibitor-1 (PAI-1), thereby negatively regulating brain endothelial fibrinolysis [31].

Until now, many transport systems have been discovered that play an important role in maintaining BBB integrity and brain homeostasis but also influence drug transport to the brain. These comprise carrier-mediated transport (CMT) and receptor-mediated transport (RMT) systems including cationic and anionic influx

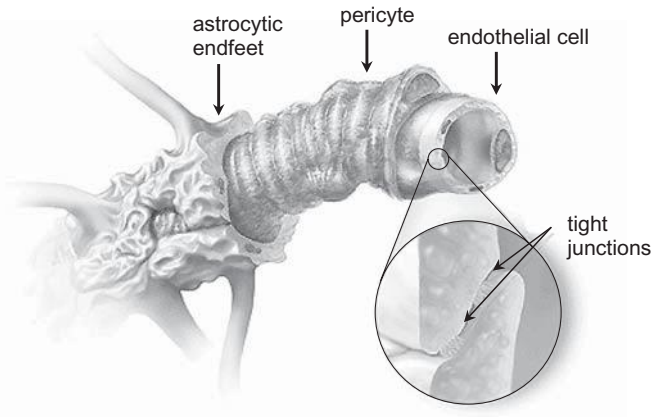


FIGURE 11.2 Schematic representation of surrounding pericytes (covering about 20–30% of the capillary surface) and astrocytic endfeet projecting on the endothelial cells of the cerebral capillaries that induce and maintain the blood–brain barrier. In contrast, endothelial cells of peripheral capillaries do not form a tight barrier because they lack the specific input of these brain cells. See text for details. (Reprinted with permission from G. Miller, *Science* 2002;297:1116–1118. Illustration: C Slayden. © 2002 AAAS.)

and efflux systems like P-glycoprotein [32, 33], multidrug resistance proteins [34, 35], nucleoside transporters, organic anion transporters (OATs), organic cation transporters (OCTs), large amino acid transporter, and the RMT systems like the transferrin-1 and -2 receptors, the melanotransferrin receptor [27], and the scavenger receptors SB-AI and SB-BI [36–38].

Because of the complex interactions between cell types and the transport of compounds to and from the brain, as well as the dynamic regulation of the BBB properties (e.g., receptor expression, formation of tight junctions), the "multitasking" BBB [39], or the "neurovascular unit" [5, 40], is considered to be an organ protecting the brain [36]. In addition, these properties are influenced by disease and drug effects, which will change the functionality of the BBB under such conditions and will influence drug delivery to the brain [6, 7, 41].

11.2.2 Blood–Cerebrospinal Fluid Barrier (BCSFB) and Ependyma

The BCSFB is a rather complicated system (see other reviews also [42–44]). It comprises mainly the choroidal and arachnoidal epithelium giving access to the ventricular and subarachnoidal CSF, respectively. Considering its location and the direction of the CSF flow, the choroidal epithelium at the choroid plexuses (CPs) can be considered to be the most important part of the BCSFB, which are located in the lateral ventricles and in the third and fourth ventricles. The CP functions as a physical, an enzymatic, and an immunological barrier and plays a role in drug metabolism, drug transport, repair, and signaling. It presents phase I–III enzymes [43]. Phase I enzymes are used for functionalization of drugs and include cytochrome P450 isoform (CYP2B1,2) and monoamine oxidase. Phase II enzymes are used for conjugation of drugs and include glutathione and glucuronosyl transferases. Phase III enzymes include transporters like Na-dicarboxylate cotransporter, the ascorbic acid trans-

porter, the organic anion transporter (OAT), the organic anion transporter polypeptide 1 and 2 (OATP1,2), the organic cation transporter (OCT), equilibrating and concentrative nucleotide transporters (ENTs and CNTs), and multidrug transporters like P-gp and MRP1 [4, 33, 34, 36]. Furthermore, the CP seems to be involved in repair by secreting neuroprotective compounds and acting as a site of neurogenesis [43]. In addition, the CPs express many receptors and several of them are involved in signaling between the immune system and the brain ([46]) and pathology of the CP has been found in many CNS disease conditions [43].

In humans the total volume of the CSF is about 160 mL and the formation rate is about 0.35–0.40 mL/min. It is produced mainly by the CPs (60%) and the other part (40%) via ultrafiltration by brain capillaries [47]. It flows from the lateral to the third and fourth ventricles and subsequently into the cisterna magna and other large basal cisterns (see Fig. 11.1). From there the CSF flows posterior and downward into the subarachnoid space around the spinal cord and upward around the cerebral hemispheres. Via the arachnoidal villi in the subarachnoidal space the CSF flows outward into venous blood.

Since the endothelium in the blood capillaries in the CP is fenestrated, resistance to drug transport seems to be presented by (gap) junctions of the CP epithelium, which are more permeable than the tight junctions of the BBB endothelium [42]. Moreover, blood flow in the CP blood capillaries seems to be 5–10 times higher than the mean cerebral blood flow [43, 46, 48]. In addition, it has been calculated that the total surface area of all CPs is of the same order of magnitude as the entire BBB [13]. All these facts together allow for the possibility of substantial drug transport via the CPs into the CSF. However, as mentioned before, the surface area of the BCSFB is facing the CSF and not the blood, which makes drug transport to the CSF less effective. Moreover, from the point of view of drug transport to the brain parenchyma, the BCSFB has some disadvantages. Once a drug is in the CSF, an additional barrier for the transport of molecules is presented by the ependyma covering the ventricles. The ependyma is a single layer of epithelial cells connected by (gap) junctions and seems to be rather permeable, particularly to small molecular lipophilic compounds, while permeability for macromolecular drugs has been claimed (see also in Section 11.4.1, Direct Injection/Infusion of Macromolecular Drugs) [42].

11.3 METHODS TO MEASURE DRUG TRANSPORT TO THE BRAIN

11.3.1 *In Vitro* Methods

Research on drug transport across the BBB and BBB functionality has been very much enhanced by the availability of *in vitro* BBB (co)culture systems and many attempts have been made to develop such systems [49–60]. In addition, the use of such systems allows a detailed investigation of BBB related phenomena at the (sub)cellular level in the absence of feedback systems from the rest of the body. This makes it much easier to study *in vitro* BBB transport and BBB functionality by (pharmacological) intervention techniques like the application of receptor agonists and antagonists, blockers of transporters and enzymes, interference RNA and (anti)gene approaches, and the influence of disease.

BBB (co)culture systems comprise primary isolated cells (including their isolation procedures) or cell lines, the use of endothelial cells alone or cocultured with astrocytes (rat fetal or C_6-glioma), the application of epithelial cells as a BBB model, and the use and availability of human cell lines [61]. One of the most important issues is the variability and transference of data between laboratories [62] and the pitfalls in performing experiments and evaluating permeability data [63].

Primary Isolated Cells

Comparison of Isolation Procedures Preparation of the *in vitro* BBB from primary isolated cells involves the isolation of capillaries and culture of BCECs alone or in combination with astrocytes or astrocyte conditioned medium. The first step in the culture of BCECs is the isolation of brain capillaries or brain capillary fragments. These can be isolated from human, bovine, porcine, and rat brain by various procedures, all starting with the grey matter of the brain [64]. Although human material would be most ideal from a scientific point of view, it is less easily available, and therefore most routinely applied procedures start from animal brains. Preferentially, bovine or porcine brains are used because of their larger mass and therefore the large amount of brain capillaries that can be isolated. The isolation procedures that have been used most frequently can be classified into nonenzymatic mechanical, combined mechanical–enzymatic, or enzymatic procedures [65–69]. The typical main steps in the various procedures are shown in Table 11.1.

The *enzymatic procedure* [65, 66] is rather time consuming (about 8 h). Following mincing of the brains into 1–2 mm cubes, two enzymatic and two separation steps are performed to isolate brain capillaries. First, an incubation step is performed with dispase followed by a dextran centrifugation/separation; and second, an incubation step with dispase/collagenase is followed by a Percoll gradient separation.

The *mechanical procedure* [55, 67] is fast and lasts 1–2 h for the isolation of capillaries from bovine brain. The brains are homogenized using two different potters. Subsequently, two filtration steps are applied: first, one isolating the material that stays behind on a 150 μm mesh, and second, one that collects the material, including capillaries that go through a 200 μm mesh.

The *combined mechanical–enzymatic procedure* [68, 69] lasts for about 4 h. It comprises the homogenization of brains by a Wheaton homogenizer followed by a filtration step through a 150 μm nylon mesh. The trapped material is subsequently treated with collagenase/DNAse and filtered through a 200 μm nylon mesh. The capillary fraction is collected by centrifugation and may be stored at –80 °C by freezing in a mix of fetal calf serum and 10% (v/v) DMSO.

Culture of BCECs Brain capillaries may be plated and cultured in culture flasks. The BCECs, which have grown out of the capillaries, may be isolated by (micro)trypsinization and plated in culture flasks or on (polycarbonate) porous filters. Cecchelli's group [55, 67] claims that the mechanical procedure results in pure BCEC cultures: first, because of the isolation of brain capillaries (not arterioles and venules) that is provided by a selective adhesion at an extracellular matrix of corneal endothelial cells, and second, because of a microtrypsinization that avoids contamination by other cell types. In the other two methods, collagen (human placenta type IV) and human plasma fibronectin [69] or rat-tail collagen with fibronectin [65, 66]

TABLE 11.1 Overview of the Main Steps in the Various Brain Capillary and Brain Capillary Endothelial Cell (BCEC) Isolation Procedures

Mechanical [55, 67]	Mechanical–Enzymatic [68, 69]	Enzymatic [65, 66]
Bovine brain grey matter	Bovine brain grey matter	Bovine brain grey matter
Homogenization (with two different potters)	Homogenization (Wheaton homogenizer)	Minced to 1–2 mm cubes
Filtration step 1 (<150 μm mesh)	Filtration step 1 (<150 μm mesh)	Dispase incubation (2.5 h)
Filtration step 2 (>200 μm mesh)	Collagenase/DNAse incubation (1 h)	Dextran centrifugation
Selective adhesion of capillaries at CEC[a] matrix)	Filtration step 2 (>200 μm mesh)	Collagenase/dispase incubation (4 h)
Growing BCECs	Selective adhesion of capillaries at collagen type IV)[b] and fibronectin[c] matrix	Percoll gradient
Collection of BCECs by microtrypsinization	Growing BCECs	Selective adhesion of capillaries at rat-tail collagen and fibronectin matrix
Plating BCECs and astrocytes at bottom of well	Collection of BCECs by trypsinization	Growing BCECs
	Plating BCECs and astrocytes at the other side of the filter	Collection of BCECs by trypsinization Plating BCECs

[a]CEC, corneal endothelial cell.
[b]Human placenta type IV collagen.
[c]Human plasma fibronectin.
Source: From Ref. 362, with permission.

replace the selective isolation of capillaries by the corneal endothelial cell matrix. This avoids the periodical isolation of corneal endothelial cells. The selectivity of the latter two methods is further guaranteed/enhanced by the application of either a Percoll gradient [65] or the time for attachment (2–4 h) of the capillary fragments at the collagen/fibronectin coating [69]. In addition, the application of 50% ACM in the procedure of Gaillard et al. [69] enhances the selective outgrowth of BCECs. Subsequently, in one step BCECs can be selectively collected following a short trypsinization time (<1.5 min). By this procedure one can obtain enough BCECs to get about 1000 (24 wells) filters from one isolation. The method of Audus and Borchardt [65, 66] also results in the isolation of capillaries and subsequently in a large amount of endothelial cells. In addition, the procedure of Dehouck et al. [55, 67] results in a substantial amount of BCECs to cover filters (about 300), but BCECs have to be passed at least 4–7 times. Unfortunately, passing primary cultured cells will eventually lead to a loss of BBB properties. In this respect, the preservation of properties is in our opinion an important issue and it has been shown that several can be reinduced by astrocytes [70–72].

The mechanical isolation procedure reduces the risk of losing surface molecules, which can occur during enzymatic treatment, particularly when aspecific proteases are applied and when these are not reinduced. This risk is less following collagenase treatment since this is a more selective enzyme. Therefore, induction of surface molecules is particularly important for enzymatically isolated cells, since it helps to reinduce BBB properties/molecules. In addition, it may be worthwhile to know that endothelial cells have the special property to transdifferentiate from one phenotype to another by the influence of environmental factors [73].

Importance of Astrocytes in BBB Culture Systems BCECs may be grown to confluency alone or in the presence of ACM or astrocytes. The use of astrocytes or ACM enhances the expression of various surface molecules and transporters and increases paracellular tightness [49–51, 68, 70–72].

Most frequently, astrocytes isolated from newborn rats and rat C_6-glioma cells have been used. Together with bovine or porcine BCECs, one obtains xenogenic coculture systems, which are very useful in studying drug transport and BBB functionality. In addition, preliminary data from our lab show that it is possible to construct an allogenic system of bovine BCECs with bovine astrocytes. However, these astrocytes were obtained from brains of 6–8 month old calves, which grow significantly slower than astrocytes of newborn rats and prolong culture time. A rat allogenic BBB coculture system has been established comprising rat BCECs and fetal rat astrocytes [74], and a mouse syngenic BBB coculture model has also been developed [75]. However, the disadvantage of these systems is that the amount of endothelial cells that can be obtained from mouse and rat brain is rather small in comparison to bovine and porcine brain and therefore these procedures are not yet suitable for large scale production of cocultures.

Astrocytes can be seeded at the bottom of (mostly polycarbonate) porous filters and at the bottom of the well. This may give rise to differences in endothelial functionality since factors released by the astrocytes influence BBB endothelial cells [67, 70–72]. Therefore, to study BBB functionality, particularly under acute stress conditions, endothelial cells and astrocytes should be in contact or in close approximation to each other for an optimal feedback. This is less necessary in studying only endothelial permeability of drugs. However, it may be argued that due to pharmacodynamic effects of drugs, increased or decreased endothelial permeability may be found caused by a less adequate feedback by astrocytes and astrocytic factors.

Other Cells in BBB Cultures Other cells that may occur following isolation of BCECs are pericytes and fibroblasts. Pericytes are normally present at the BBB and are surrounded by a basement membrane and have been shown to induce separately specific enzymes in BCECs [76]. In addition, it has been argued that pericytes are necessary to establish an *in vivo*-like BBB cell culture model [31]. Fibroblasts are present in brain tissue, but not very much is known about their influence at the BBB. Other cells may also have an important action in BBB cell culture systems, such as neurons, microglia, and monocytes, and it may be argued that combinations of these cells should be present in a BBB cell culture system for optimal functionality. This has been described for an *in vitro* BBB model comprising human BCECs, human fetal astrocytes, human macrophages, and neuronal (human teratocarcinoma) cells [77]. Other systems have been developed comprising cultured monolayers of brain

endothelial cell lines or selected epithelial cell lines, combined with astrocyte and neuron cultures that present a novel three-dimensional technique for the screening of neurotoxic compounds [78].

As mentioned before, human material is rather difficult to obtain regularly in large quantities. A good alternative is the use of human umbilical vein endothelial cells (HUVECs). These can be rather easily obtained and when cultured under proper conditions, such as with astrocytes, a human-like BBB system may be obtained [79]. Alternatively, human endothelial cells from skin capillaries can be used in combination with postnatal rat astrocytes [80]. Such systems are convenient for several purposes (such as transcellular lipophilic/hydrophilic transport and carrier/receptor-mediated transport) and are easy to obtain in large quantities. Still, it may be expected that the functionality of such cells is different from primary cultured BCECs (with respect to tightness, up/downregulation of transporters, etc.) although this has not been demonstrated until now.

Application of Barrier Enhancers It has been shown that several compounds like cAMP, phosphodiesterase inhibitors, glucocorticoids, and interferon-α,β may be used to enhance the tightness of BBB endothelial monolayers [68, 69, 81, 82]. In addition, the application of basic fibroblast growth factor (bFGF; secreted by astrocytes [83, 84]) is important in growing/establishing functional and tight BBB cell culture systems [55] and in the induction of BBB properties [84].

Application of these enhancers has increased the ease of culture and the properties of the cell culture system. However, apart from the lack of functional influence of astrocytes, the question that still has to be answered is how these compounds influence other aspects of BBB functionality in addition to endothelial tightness.

Endothelial Cell Lines Various immortalized brain capillary endothelial cell lines have been derived. These are made by transfection of these cells with an immortalizing gene. An overview of the most frequently used cell lines is given in Table 11.2. These cell lines have various properties that resemble *in vivo* BBB capillary endothelial cells. The advantage of cell lines is their ease in culture, their purity, and the fact that there is no need for a periodic isolation of capillaries from brains. Since these cell lines are obtained by immortalization, it may be expected that their functionality will be changed compared to primary cultured BCECs. This is illustrated by the fact that most of these cell lines are rather permeable (paracellular leakiness), which limits their use in studying BBB transport. The application of these cells is particularly useful for (immuno)histochemical identification of extra- and intracellular molecules, for studying morphological aspects of endothelial cells, and so on. In addition, barrier enhancers (see also previous paragraph) may be used to improve the tightness of these monolayers. Recently, a human brain immortalized endothelial cell line was established by transfection of the human telomerase or SV40 T antigen. This cell line (hCMEC/D3) represents a stable, well characterized, and well differentiated human brain endothelial cell line and offers opportunities for BBB research [61].

Tumor Cell Systems The application of rat C_6-glioma cells makes the culture of astrocytes much easier. This provides the same advantages as with cell lines. Table 11.3 gives an overview of the various sources of astrocytes and their application in

TABLE 11.2 Overview of the Used Immortalized Brain Capillary Endothelial Cell Lines

Cell Line	Source	References
SV-BEC	Bovine	100
t-BBEC-117	Bovine	101
BBEC-SV	Bovine	102
RBEC1	Rat	103
TR-BBB	Rat	104
RBE4	Rat	105
CR3	Rat	106
RCE-T1	Rat	107
GPNT	Rat	108, 109
GP8.3	Rat	110
TM-BBB	Mouse	111
MBEC	Mouse	112
S5C	Mouse	113
PBMEC/C1-2	Porcine	114
SV-HEC	Human	115
BB19	Human	116
HBEC-51	Human	117

Source: From Ref. 363, with permission.

TABLE 11.3 Astrocytes Used in Coculture with Endothelial or Epithelial Cells of Various Sources

Astrocyte	Coculture with Endothelium[a]
Newborn rat astrocyte	BCECs (bovine) [55, 67, 69]
Newborn rat astrocyte cell line (A_7)	BCECs (bovine) [118]
C_6-rat glioma	BCECs (bovine) [119]
C_6-rat glioma	HUVECs (human) [79]
C_6-rat glioma	RBE4 (rat) [120]
C_6-rat glioma	Aortic (bovine) [121]
TR-AST (rat)[b] [122]	
Newborn mouse astrocytes	BCECs (mouse) [75]
Human fetal astrocytes	BCECs (human) [77]
Astrocyte	**Coculture with Epithelium[a]**
C_6-rat glioma	ECV304 (human) [81]
C_6-rat glioma	MDCK (dog) [80]
Human 1321N1 astrocyte cell line	ECV304 (human) [81]

[a]HUVECs, human umbilical vein endothelial cells; RBE4, rat brain immortalized capillary endothelial cell line; ECV, T24 human bladder epithelial carcinoma cell line; MDCK, dog kidney epithelial cell line; BCECs, brain capillary endothelial cells.
[b]Conditionally immortalized astrocyte type II.

Source: From Ref. 362, with permission.

BBB coculture systems. The application of tumor astrocytic cell lines introduces the consequence of a tumor phenotype in BBB cocultures. This may be very helpful if one is interested in the blood–tumor barrier in the brain because the homeostasis of the tumor–BBB endothelium may be different. Surface molecules and transport-

ers may be up- or downregulated and may give leads for tumor targeting. These arguments make these BBB systems more suitable to study only passive hydrophilic or lipophilic drug transport. Since active transport processes may be differentially regulated under tumor conditions, the results of such transport studies are less relevant for those under "healthy" BBB conditions. Therefore, these tumor BBB coculture systems are not suitable to study active transport processes and functionality under other than tumor conditions. Apart from the tumor phenotype, these BBB coculture systems differ with respect to the source of the endothelial cells, while the ECV304 (recently characterized as a T24 bladder epithelial carcinoma cell, see [58]) and MDCK (dog kidney epithelial cell line) cell lines represent epithelium. It has recently been shown that the basal transendothelial electrical resistance (TEER) of ECV304 monolayers could be enhanced by human 1321N1 astrocytes and primary rat astrocytes and that relatively tight monolayers could be obtained [81]. However, the replacement of endothelial cells by epithelial cells may have some consequences. In the first place, endothelial cells are longitudinal cells with a thickness of 2–4 μm in contrast to MDCK cells that are columnar cells with a thickness of more than 20 μm. Second, (drug) metabolism by epithelial cells may be quite different from endothelial cells; and third, transporters may be up- or downregulated and absent or mutated.

Variability and Transference of Results Between BBB Culture Systems BBB culture systems have been the subject of a Concerted Action within the EEC entitled *Drug Transport Across the BBB: New Experimental Strategies*, involving 21 research groups from 9 European countries [85]. The focus of this Concerted Action was on the optimization, harmonization, and validation of brain-microvessel-endothelial cell culture and microdialysis to develop and study new experimental strategies for drug transport to the brain. The various BBB culture systems have been described in the report of this Concerted Action including the sources of variability [62]. Furthermore, pitfalls in evaluating BBB data should be noted in order to avoid bias and misinterpretation of results [63]. Here we want to indicate and compare the various properties of the most frequently applied BBB (co)culture systems. In addition, others have indicated the need for validated BBB culture systems [86, 87]. It has been discussed elsewhere that the transference of data from various BBB culture systems and the evaluation of the results would be enhanced if these were obtained in a well validated context [62]. Presently, there is no laboratory-to-laboratory validation of BBB systems and only individual researchers characterize and validate their BBB systems by the expression of various surface molecules and enzymes, paracellular marker molecules (^{14}C-mannitol, ^{14}C-sucrose, fluorescein, and FITC-dextran MW 4000), and the measurement of the transendothelial electrical resistance (TEER) [82, 88]. In addition, since TEER values measured before the experiment do not guarantee BBB tightness during the experiment, this method is very helpful to estimate BBB performance during and after the experiment also. Efforts have been focused on the use and validation of *in vitro* BBB systems to estimate BBB transport of drugs and on the *in vitro*/*in vivo* correlation of BBB drug transport by the ECVAM (European Center for Validation of Alternative Methods) [89, 90].

Furthermore, the misinterpretation of results due to biased *in vitro* conditions may be a serious point [63]. Physiologically relevant *in vitro* conditions (pH, serum

proteins, unstirred water layer, cellular retention, and use of artificial membranes) should be established as much as possible and recommendations have been made to optimize *in vitro* systems accordingly [63].

Application of BCEC Cocultures in Research BBB (co)cultures are useful to study a whole variety of phenomena. These include transport processes like passive hydrophilic (paracellular) transport, passive lipophilic transport, carrier- or receptor-mediated (transcellular) transport, and fluid-phase and adsorptive-mediated transport. The discovery of efflux transporters like P-glycoprotein and MRPs (reviewed in [34–38, 91]) and influx transporters like the amino acid, the monocarboxylic acid, the cationic drug, the hexose, the nucleotide, and the peptide transport systems (reviewed by [92]) have contributed much to our understanding of BBB transport. In addition, drug metabolism [22–24], the effect of specific agents and conditions on the functionality of the BBB including disease state, the visualization of drug transport routes by confocal laser scanning microscopy (CFLM) [93], and the measurement of the transendothelial electrical resistance [80] can be studied.

Particularly, disease state is an important BBB condition to be studied, since it has been shown to change BBB functionality and therefore drug transport to the brain [80]. In addition, inflammatory conditions, which may occur in multiple sclerosis, Alzheimer disease, AIDS-related dementia, meningitis, and encephalitis, can be induced at the *in vitro* BBB by applying lipopolysaccharide (LPS), TNF-α, IL-1β, and NO [6, 7, 9, 75]. Under such disease conditions, BBB permeability, functionality, and drug transport can be studied together with (pharmacological) intervention. The application of functional genomics and the phage-display technique to BBB coculture systems is in this respect very interesting in order to identify transcribed genes and expressed surface molecules under various conditions including disease state.

Conclusions The presently available *in vitro* BBB (co)culture systems have their specific properties and can be used for specific goals (see Table 11.4). These goals can be passive hydrophilic and lipophilic drug transport, the study of active transport processes, and BBB functionality. It may be clear that for the study of passive drug transport through the BBB, sophisticated BBB systems are not required. However, since monolayers may be leaky, a wrong interpretation may be obtained on the contribution of para- and transcellular transport to the total transport and corrections should be made [94]. BBB coculture systems that resemble as much as possible the *in vivo* BBB must be used when active transport processes and the study of BBB functionality are involved. Tumor (co)culture systems are more useful to study the tumor BBB, while other systems [67, 69] are more useful to study active BBB processes and BBB functionality under healthy and disease conditions.

The influence of BBB pathology and the influence of disease mediators like LPS, NO, and radical oxygen species/radical nitrogen species (ROS/RNS) on BBB permeability is well known, particularly with respect to paracellular permeability [6, 7, 9, 75]. In addition, disease may up- or downregulate active transcellular transport systems (transporters and transcytosis mechanisms). Less is known about the influences of the pharmacodynamic effects of drugs at the BBB, which may increase (e.g., glucocorticoids) [82, 95] or even decrease (doxorubicin, vinblastine given together

TABLE 11.4 Overview of the Various *In Vitro* BBB Coculture Systems Involving the Use of C_6-Rat Glioma Cells

E/A Coculture	Useful for Study of	Not Ideally Suited for Study of	References
Endothelium/astrocyte coculture			
HUVEC (human)/ C_6-glioma (rat)	L; H; CLSM	BBB-F; BBB-D; DT; DM; DE	79
BCEC (human)/ human fetal A	L; H; CLSM; DT; DM; DE "human"-BBB; BBB-F; BBB-D		77
BCEC (bovine)/ newborn (rat) A-cell line	L; H; CLSM	BBB-F; BBB-D; DT; DM; DE	118
BCEC (bovine)/ newborn rat A	L; H; CLSM; DT; DM; DE "normal"-BBB; BBB-F; BBB-D		67, 69
BCEC (mouse)/ newborn mouse A	L; H; CLSM; DT; DM; DE "normal"-BBB; BBB-F; BBB-D		75
BCEC (bovine)/ C_6-glioma (rat)	L; H; tumor-BBB; CLSM	BBB-F; BBB-D; DT; DM; DE	119
RBE4 (rat)/ C_6-glioma (rat)	L; H; tumor-BBB; CLSM	BBB-F; BBB-D; DT; DM; DE	120
Epithelium/astrocyte coculture			
MDCK (dog)/ C_6-glioma (rat)	L; H; CLSM	BBB-F; BBB-D; DT; DM; DE	123
ECV304 (human)/ C_6-glioma (rat)	L; H; CLSM	BBB-F; BBB-D DT; DM; DE	81
ECV304 (human)/ 1321N1 (human)	L; H; CLSM	BBB-F; BBB-D DT; DM; DE	81

Abbreviations: E/A, endothelial or epithelial coculture with astrocytes; A, astrocyte; L, passive lipophilic transport; H, passive hydrophilic (paracellular transport); BBB-F, BBB functionality; BBB-D, BBB diseases; DM, drug metabolism; DE, drug effects; DT, BBB drug influx and efflux transport systems; CLSM, confocal laser scanning microscopy.

Source: From Ref. 362, with permission.

with a P-gp inhibitor) paracellular tightness [72, 96, 97]. In addition, several diseases in the brain (multiple sclerosis, Alzheimer disease, AIDS-related dementia, meningitis, epilepsy, tumors, etc.) are the primary or secondary cause of BBB permeability. Therefore, it is important to consider the pharmacodynamic (therapeutical or toxicological) effects of drugs at the BBB also.

Variability [62] and physiologically relevant *in vitro* conditions [63] are probably the main obstacles to the transference of results between laboratories. An interesting example is the expression of P-glycoprotein in BCECs depending on cell culture conditions. Barrand et al. [98] have shown that mdr1a–P-gp in cultured rat BCECs was downregulated and changed into the expression of mdr1b–P-gp. In addition, it has been shown that P-gp expression in bovine BCECs could be upregulated again by rat astrocytes [72]. Similar data have been published by Zhang et al. [99], where the expression of MRPs (at mRNA level) in brain capillaries was qualitatively and

quantitatively different from those in BCEC monolayers. Therefore, it may be stressed again, that the systems to be used are well characterized morphologically, biochemically, and functionally. Together with the use of validation markers [86] and physiologically relevant *in vitro* conditions, this will lead to an optimal transference of BBB data between laboratories [62, 63, 89, 90].

11.3.2 *In Vivo* Methods

Although the *in vitro* systems have evolved into sophisticated and functional models of the BBB, they may result in quantitative and qualitative differences in BBB transport due up- or downregulation of transporters and species differences. Therefore, it is necessary to verify and validate the obtained *in vitro* data *in vivo*. This requires the application of animal-based methods. There are several methods; however, their applicability depends on the sensitivity and selectivity to measure drug concentrations in the brain of fast and poor BBB/brain penetrating compounds, the estimation of local concentrations in the brain or whole brain distribution, and the measurement of single-time concentrations versus concentration time profiles. Therefore, it is essential to understand the possibilities and limitations of these methods for the estimation of BBB transport including the role of drug influx and efflux transporters at the BBB [36].

Here we briefly discuss the following methods in the context of their possibilities and limitations to estimate BBB transport: brain uptake index (BUI), multiple pass techniques, brain efflux index (BEI), *in situ* brain perfusion, CSF (cerebrospinal fluid) sampling (unit impulse response), positron emission tomography (PET), magnetic resonance techniques (imaging (MRI), spectroscopy (MRS)), quantitative autoradiography (QAR), and intracerebral microdialysis. Most of these methods are invasive in contrast to the noninvasive ones like PET and NMR.

Brain Uptake Index (BUI) This is one of the oldest techniques (1970) to estimate the uptake (via the BBB and/or blood–CSF barrier) of drugs/compounds into the brain [124, 125]. The method is widely used, rapid, and relatively cheap and the surgery is easy. It is a single passage technique and comprises the intra-arterial administration (common carotid artery) of a solution containing a reference (mostly a ^{14}C-labeled compound) and a test compound (preferentially a ^{3}H-labeled compound) under anesthesia. The basic idea is that the reference compound can easily penetrate the brain and the test compound less. Following a short time (15 s) the animal is decapitated and brain concentrations of test and reference compounds are measured and related to the plasma concentration. This ratio is a measure for the brain uptake of the test compound that is expressed as a percentage of the penetration of the reference compound. This procedure is very suitable for compounds that are labile or fast metabolized. Its drawback is that the exposure to the brain is very short and results from BUI studies cannot easily be related to cerebrovascular permeability. These are dependent on blood flow, brain region, and time between injection and decapitation. Therefore, this procedure is not suitable for poorly penetrating compounds including peptides and proteins. Another limitation is that the total injected dose does not reach the whole brain since about 20% of the drug enters the internal carotid artery while 80% reaches the external carotid artery.

Brain Efflux Index (BEI) The BEI has recently (1996) been developed [126, 127]. It comprises the estimation of the efflux of compounds from the brain following microinjection (maximal 1 μL) at a specific site in the brain, compared to a reference compound (^{14}C-carboxy-inulin) that has a limited BBB permeability. Following decapitation at variable times, the brain and plasma concentration of compound and reference can be estimated. The BEI is expressed as the percentage of the ratio of drug effluxed from the brain and the drug injected into the brain. The apparent elimination rate constant can be calculated by plotting the log(BEI) versus time. Subsequently, the efflux clearance of compounds from the brain can be calculated. The technique is cheap and the surgery is moderately easy.

Multipassage Techniques These techniques have been developed to circumvent several of the drawbacks of the BUI method and were originally designed by Brodie et al. [128]. The exposure is much longer and thus measurable brain concentrations of poorly penetrating compounds can be obtained. Later on, mathematical models were developed to calculate the permeability surface area (PS) product after an intravenous bolus administration [129]. Generally, the concentration of the drug in the brain is divided by the integral of the plasma drug concentration and time (AUC). This results in the estimation of the clearance of drug from blood or plasma to brain and is expressed as the PS product for BBB and/or BCSFB transport. For an accurate estimation of the PS of slowly penetrating compounds, it is essential to correct for the amount of drug in the vascular bed of the brain. Furthermore, it has been speculated that the PS product was time dependent [130].

The multiple-passage/multiple-time-point techniques provide information about the time course of brain uptake [128, 131, 132]. Originally, these experiments were performed by maintaining plasma steady-state concentrations and following the time course of brain uptake by sacrificing groups of animals at different time points. BBB transport can be estimated when the concentration difference between brain or CSF and plasma relative to the plasma concentration is plotted versus time. Later on, this procedure was further developed to intravenous bolus administration [133]. Ultimately, the PS products can be calculated by the Renkin–Crone equation [134]. The interesting advantage of this equation is that the amount of drug penetrating the brain can also be estimated by positron emission tomography (PET) and computer tomography (CT) scans, which allow a noninvasive measurement of BBB permeability in humans [135]. An additional advantage is that the regional blood volume does not have to be estimated. However, since brain sampling can only be done after sacrificing the animal, it is not possible to study the time profile of BBB transport in a single animal. The technique is cheap and the surgery easy to perform.

In Situ Brain Perfusion Techniques These were developed (1984) to avoid (non-brain) metabolism of the test compound and to fully control the passage of the BBB and/or the BCSFB. These techniques do not require estimation of the regional blood volumes to correct brain concentrations. In this technique the common carotid artery is ligated and the (^{14}C-labeled) reference and (^{3}H-labeled) test compounds are perfused retrograde via the external into the internal carotid artery under anesthesia [136]. Following perfusion the animal is decapitated and the brain is collected and analyzed for reference and test compounds. The use of two test compounds (a

highly diffusible one and the other an intravascular marker compound) allows an accurate estimation of the PS product [129]. The problem with this technique is that in rats the common carotid artery has branches between the neck region and the brain. Therefore, to ensure the delivery of compounds to the brain, these branches have to be ligated. Guinea pigs do not have these branches and are therefore more convenient animals to use in this respect [137]. The technique is cheap and surgery is easy.

Unit Impulse Response Procedure This procedure together with numerical deconvolution [138–140] has been applied (1989) to estimate BBB and/or BCSFB transport based on serial CSF sampling in the *cisterna magna* [141]. This procedure allows the construction of a complete time–transport profile in a single anesthetized animal. It requires the estimation of a weighing function comprising the characterization of the disposition of a drug in the brain. This is achieved by injecting a compound in the lateral ventricle of the brain, followed by sampling of the CSF. Subsequently, the compound is given intravenously and plasma and CSF samples have to be collected. The BBB input profile can be obtained by a mathematical procedure called deconvolution [139, 140]. Principally, one can obtain information on passive and active transport processes in the brain. Essentially, the cumulative cleared plasma volume is plotted against time. When a straight line is obtained, its slope represents the uptake clearance. In the case of nonlinearity, the V_{max} and K_m can in principle be estimated. The method has been applied to lipophilic drugs like atenolol, acetaminophen, and antipyrine [141]. The advantage of this technique is that it avoids the occurrence of backdiffusion from brain to blood. The costs of the technique and the complexity of the surgery are moderate.

Positron Emission Tomography (PET) Regional BBB and BCSFB permeability and drug transport can be investigated by PET [142]. This technique employs the intravenous administration (bolus or infusion) of positron emitting isotopes like ^{18}F, ^{13}N, ^{11}C, or ^{15}O. By this technique brain and plasma concentrations can be followed over a long time. Subsequently, one analyzes the data by pharmacokinetic models that describe the transport of tracers (uptake, distribution, and elimination). In 1955, this technique was developed by Davson [143]. The advantage of the technique is that the transport of tracers can be visualized and studied in whole brain over time. However, the technique is expensive and the preparation and stability of the tracers are matters of concern. Essentially, tracer concentrations are measured and no distinction can be made between parent compound, metabolite, and protein bound or unbound tracer. Nevertheless, this technique is sensitive and therefore very helpful in diagnostic purposes such as the localization of tumors in the brain and also in studying local differences in BBB permeability and BBB transport. P-gp functionality has been investigated with ^{11}C-verapamil in mdr1a (+/+) and (–/–) mice [142, 144]. The PET procedure underestimated the uptake of ^{11}C-verapamil in the brain. However, in patients, pretreatment with cyclosporin A increased ^{11}C-verapamil levels in GLC4/P-gp tumors (184%) and in brains (1280%). This pharmacokinetic effect was clearly visualized with PET [145].

Quantitative Autoradiography (QAR) This method was initially applied in 1951 by Campbell [146] and later on used to visualize the distribution of radioactive

tracers across the BBB and/or the BCSFB [131]. Typically, these are ^{3}H- of ^{14}C-labeled compounds that are given intravenously. Following administration, the animals are sacrificed after various times, brains collected, and slices prepared. Subsequently, autoradiographs are prepared that can be scanned by a computer driven densitometer. The technique is cheap and easy to perform. The spatial resolution of QAR is high; however, the time resolution is rather low since one animal has to be used for one time point. Other drawbacks are that no distinction can be made between parent compound and metabolites, and protein bound and unbound compounds.

Intracerebral Microdialysis This method was developed in the late 1950s and measures particularly local concentrations of compounds in the extracellular fluid (ECF) of the brain or the CSF. It is an invasive method and longitudinal, I-shaped or semicircular probes can be used [147]. The time to perform experiments has to be evaluated carefully with respect to periprobe tissue reactions that may occur [148]. The method has been successfully applied in the estimation of BBB transport of various compounds [148–151]. The advantage of this method is that concentration–time profiles can be obtained of unbound compounds in the extracellular fluid in the brain. The spatial resolution of this technique is relatively high, and a highly sensitive and selective analytical technique is essential for estimation of concentrations of compounds in the ECF. Presently, HPLC combined with mass spectroscopy methods result in high selectivity and sensitivity. The surgery is moderately difficult and the costs are moderate.

An essential factor is the estimation of the *in vivo* recovery of the microdialysis probe. This describes the relation between the concentration of the compound in the fluid around the probe and that in the dialysate. *In vitro* recovery is mainly dependent on the perfusate composition and flow rate, the physicochemical characteristics of the compound, temperature, and probe characteristics. In addition, *in vivo* recovery is influenced by the para- and transcellular transport of compounds through brain tissue and the blood supply in terms of vascularization and blood flow [147]. *In vivo* recovery can be estimated by the "nonet flux" (measurement of recovery under serial steady-state conditions in one animal) [152] and the "dynamic nonet flux" (measurement of recovery under one but different steady-state conditions in different groups of animals) [153] methods and by the internal standard, the retrodialysis, and reverse dialysis methods (reviewed in Ref. 147).

Intracerebral microdialysis allows the estimation of the local BBB time transport profile of unbound compounds in the ECF of the brain and in the CSF. It can distinguish between parent compound and metabolite(s). The analytical technique to estimate the substances in the ECF or CSF must be very sensitive and selective. In addition, the conversion of dialysate concentrations to real extracellular concentrations in the brain is very much determined by the procedure to estimate *in vivo* recovery.

Magnetic Resonance Techniques These comprise magnetic resonance imaging (MRI, initially applied in the late 1970s) and magnetic resonance spectroscopy (MRS, initially applied in the 1950s) and are noninvasive techniques. They do not require the use of ionizing radiation or the use of iodinated contrast compared to X-ray computed tomography (CT). They comprise the direct or indirect monitoring

and recording of the spatiotemporal distribution of molecular or cellular processes for biochemical, biologic, diagnostic, or therapeutic applications [154]. The techniques employ ^{1}H-, ^{13}C-, and ^{31}P-labeled compounds that are injected intravenously. This allows evaluation of a variety of parameters in the brain related to anatomy, physiology, and metabolism, like macrophage infiltration, cytotoxic edema, cerebral blood flow and volume, and metabolic processes [155]. Depending on the technique used, slice-based as well as true 3D images can be obtained [156]. MRI techniques have been developed into functional MRI (fMRI) techniques, which measure regional blood volume and flow, regional diffusion of water (diffusion tensor imaging—DTI) [157, 158], and regional blood oxygenation level dependence (BOLD) [159, 160]. In addition, when combining a contrast enhancing principle (superparamagnetic iron oxide—SPIO; Mn^{2+}, Gd^{3+}) [160] with a target-specific carrier moiety (receptor ligand, antibody, cell), highly specific information on targeted molecular interactions can be obtained by molecular (brain) imaging [161]. This is very important from the diagnostic and therapeutic (e.g., disease progression) point of view. However, the problem is to deliver these agents through the BBB, and successful strategies have to be developed to accomplish this. Nevertheless, MRI has been used to investigate BBB permeability and leakage involved in brain diseases including neurodegenerative diseases [157, 162]; various brain tumors [163, 164], multiple sclerosis [165, 166], early infarcts, and inflammatory lesions can be studied [157] in addition to age related changes in brain tissue [158]. Moreover, since there are many disease models in small animals, MRI can be used to study these (brain) disease processes dynamically [156].

MRS has been used successfully to quantify phenylalanine (Phe) concentrations in the brain of patients with phenylketonuria and to estimate its BBB transport (PKU) [167]. In addition, it was shown that BBB transport parameters determined the clinical outcome in phenylketonuria [168]. In studies with multiple sclerosis (MS) patients, it was demonstrated that the earliest detectable event in the development of a new lesion was the increase in permeability of the BBB that was associated with inflammation [169]. It was shown that when the inflammation declined, edema resolved. With ^{1}H-MRS a correlation was shown between a decrease in *N*-acetylaspartate (NAA) concentrations (a marker of neuronal or axonal damage) and clinical disability [170].

With these techniques one can differentiate between parent compound and its metabolites as well as between free and unbound drug concentrations. The sensitivity and its spatial resolution are limited but resolutions ranging from 300 to 500 μm throughout the human brain and 50 to 100 μm through the rodent brain should be possible in reasonable scan times [159]. The latter are presently up to 2 hours while another disadvantage is that the technique is expensive [162]. Nevertheless, magnetic resonance techniques are very convenient to estimate BBB time transport profiles of poorly and fast brain-penetrating compounds.

Conclusions Various methods have been developed to estimate drug transport to and from the brain. Table 11.5 shows an overview of the typical properties of the reviewed methods. Presently the brain perfusion method is one the most frequently used methods in animals. It allows the estimation of brain uptake of fast and poorly penetrating compounds at single times. In addition, when more animals are used a concentration–time profile can be obtained. The BEI method is applicable in the

TABLE 11.5 Applicability[a] of Various Methods to Estimate Drug Transport and Distribution in the Brain

Method	Fast Penetrating Compounds	Poor Penetrating Compounds	Single Concentration at Single Time	Concentration–Time Course in Single Animal	Brain Distribution[b]	Costs	Surgery
BUI	+	–	+	–	–	Cheap	Easy
Multipassage techniques	+	+	+	–	–	Cheap	Easy
BEI	+	+	+	–	–	Cheap	Moderately easy
Brain perfusion	+	+	++	–	–	Cheap	Easy
Impulse response method	+	+	++	+	– (CSF)	Cheap	Moderately difficult
PET	+	+	+	++ (human)	+ (WB)	Expensive	Easy
QAR	+	+	+	–	++ (WB)	Cheap	Easy
MRI/MRS	+	+		++ (human)	+ (WB)	Expensive	Easy
Intracerebral microdialysis	+	+		+	+ (local)	Moderate	Moderately difficult

[a]+, applicable; ++, very good applicability; –, not appplicable.
[b]WB, whole brain; CSF, only in cerebrospinal fluid.

same way and allows the measurement of brain efflux of compounds. The BUI, BEI, multipassage techniques, brain perfusion, and the impulse response method (only in CSF) do not give information on brain distribution. In contrast, PET, QAR, MRI/MRS, and intracerebral microdialysis provide information on the brain concentration–time course and brain distribution of compounds in one single animal. However, the resolution varies, with that of QAR and intracerebral microdialysis > PET and MRI/MRS. In addition, QAR gives information on whole brain distribution of compounds and intracerebral microdialysis only gives information about the unbound concentration in the brain area where the probe has been implanted. Both methods are suitable for routine use in animals while intracerebral microdialysis has been used to monitor patients with brain trauma [171]. In particular, PET and MRI/MRS can be applied in humans and can allow the estimation of the concentration–time course of compounds and their distribution in the whole brain. Moreover, the advantage of MRI/MRS is that a distinction can be made between metabolites and unbound and bound drug.

11.4 STRATEGIES FOR DRUG DELIVERY TO THE BRAIN

For many diseases of the brain, such as Alzheimer disease, Parkinson disease, stroke, depression, schizophrenia, epilepsy, and migraine headache, the drugs on the market are far from being ideal, let alone curative. A significant part of the problem is the poor BBB penetration of most of the drugs in development against neuronal targets for treatment of these disorders. This includes approximately 98% of the small molecules and nearly 100% of large molecules, such as recombinant proteins or gene-based medicines [12]. Therefore, much effort is put toward delivery and targeting of drugs to the brain [10, 12, 172]. Drug delivery to the brain can be achieved via several methods, including local invasive (direct injection/infusion) delivery, induction of enhanced permeability, and the application of global physiological targeting strategies. Apart from the delivery of small molecules (e.g., <500 Da) to the brain, the global delivery of large hydrophilic molecules, including enzymes, interference RNA (RNAi), and genes, present a serious problem.

11.4.1 Local Brain Delivery by Direct Injection/Infusion of Drugs into the Brain

Because of the lack of ability to target via the vascular route to the brain, invasive methods, like direct injection or infusion and convection enhanced delivery, have been applied to macromolecular drugs as well as to the delivery of viral vectors. These are reviewed in the following paragraphs.

Direct Injection/Infusion of Macromolecular Drugs Intracerebral ventricular (ICV) or intrathecal drug infusion comprises direct injection/infusion of drug into the CSF. However, as mentioned before, CSF is completely drained into the venous circulation, and drugs still have to cross the ependymal brain–CSF barrier. This has shown to be feasible particularly for many small (mostly lipophilic [45, 172]) drugs following intraventricular administration. In addition, it was shown that compounds with MW < 5000 rapidly penetrate the ependyma but that penetration into the brain parenchyma was limited due to diffusion, tortuosity, transcapillary loss, cell uptake,

and binding [173, 174]. As a result, the infused drug has minimal access to the parenchyma by diffusion (Fig. 11.3C) [175]. In addition, it was shown that compounds with MW > 5000 very poorly penetrated the brain parenchyma even when they were administered intraventricularly. It was shown that following a 3–5 h infusion of ^{14}C-inulin in the lateral ventricle of dogs, only an appreciable uptake in tissues lining the ventricle was found [176] and intraventricular injection of ^{125}I-brain-derived neurotrophic growth factor [177] in rats resulted in a very poor uptake by the brain parenchyma. Very recently, it was elegantly demonstrated that the uptake of ^{125}I-insulin-like growth factor-1 (MW 7.7 kDa) following intraventricular infusion was very poor by brain parenchyma and was rapidly cleared from the cerebrospinal fluid compartment mainly into blood [178]. In addition, these authors stated that under healthy conditions the CSF flow direction will probably be different from that under disease conditions and that further investigations are required in this respect. Furthermore, experimental data in human parkinsonian patients indicate that intraventricular GDNF infusion by a Medtronic device was biologically active but did not improve parkinsonism symptoms, possibly because GDNF (dimer with MW 33–45 kDa) did not sufficiently reach the target tissues [179].

Direct infusion into the brain has also been applied. The delivery of GDNF by direct infusion into the putamen of parkinsonian patients in one study showed

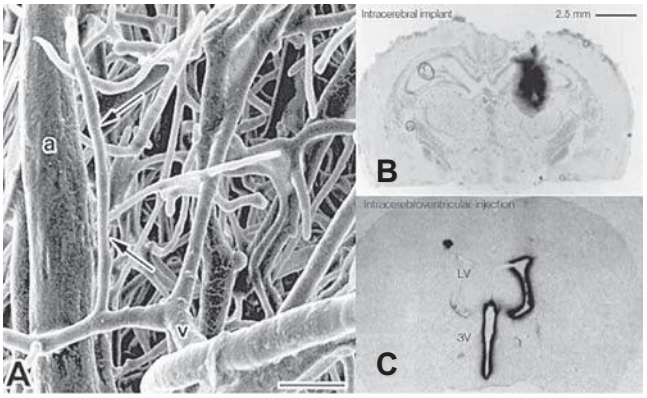


FIGURE 11.3 Drug delivery via the vascular route will enable widespread distribution of the drug to each single neuron within the brain (note that the bar in panel A indicates a length of 25 μm, which is about the size of a single neuron). (A) Scanning electron micrograph of a vascular cast of a mouse brain (a, artery; v, vein). (Reprinted from Satomi et al., Cerebral vascular abnormalities in a murine model of hereditary hemorrhagic telangiectasia. *Stroke* 2003;34(33):783–789. © 2003, with permission from Lippincott Williams & Wilkins.) (B) Minimal diffusion of [^{125}I]-nerve growth factor (NGF) after intracerebral implantation of a biodegradable polymer (note that the bar indicates a length of 2.5 mm, which was also the size of the implant). (Reprinted from Krewson et al., Distribution of nerve growth factor following direct delivery to brain interstitium. *Brain Res* 1995;680(1–2):196–206. © 1995, with permission from Elsevier.) (C) ICV injection of [^{125}I]-brain-derived neurotrophic factor (BDNF). (Reprinted from Yan et al., Distribution of intracerebral ventricularly administered neurotrophins in rat brain and its correlation with trk receptor expression. *Exp Neurol* 1994;127(1):23–36. © 1994, with permission from Elsevier.) Note that the neurotrophin does not distribute into the brain beyond the ipsilateral ependymal surface.

general improvement over time [180] while a Phase II clinical trial could only demonstrate "potential efficacy" [181]. Indeed, Amgen decided to confirm its previous decision to stop a Phase II clinical trial with GDNF infusions into the putamen of 48 patients because "scientific results indicated that allowing patients to continue treatment could potentially cause permanent harm, complicating an already devastating disease" [182].

A variant of this is *convection enhanced drug delivery*, where a positive pressure infusion in brain parenchyma has been applied to increase drug uptake [183–185]. In addition, uptake was significantly improved when the viscosity of the infusate was increased [183], which seems to be caused by a similar mechanism (osmotic opening) as has been applied by Kroll and Neuwelt [186]. Good results have been shown by large volume infusion of naked DNA in the tail vein or legs where temporarily blood flow was inhibited; however, this is not applicable to the brain [187, 188].

Furthermore, brain implants have been used for the local delivery of drugs to the brain (Fig. 11.3B). Indeed, it has been shown that polymeric implants (poly(ethylene-*co*-vinyl acetate)) containing ^{125}I-nerve-growth factor into rat brain were effective for local delivery, but unfortunately not for global delivery, of the drug [189].

In conclusion, because of the fenestrated capillaries in the CPs and their total surface area that is comparable to the BBB, drug transport into the CSF could be substantial. However, apart from the need of direct injection into brain parenchyma or infusion into the ventricles, for drugs that have to reach their target by passive diffusion, the direction of the CSF flow in the ventricles, the presence of the ependymal barrier (particularly for compounds with MW > 5000), and the direction of the flow of the interstitial fluid in the brain parenchyma are major obstacles for an effective global penetration into the brain.

Direct Injection/Infusion of Viral Vectors Gene therapy [190–193] is meant to deliver genetic material with a therapeutic function encoding proteins (e.g., enzymes) or siRNA/shRNA to somatic cells [194]. In addition, gene therapy could provide a long-term effect following one single administration. However, genes are hydrophilic and charged and are large molecules that cannot pass cell membranes and tight cellular layers like the BBB. Therefore, means have to be found and developed to transport genes to the desired site of action.

Viruses have evolved over millions of years to obtain optimal mechanisms for gene delivery to host cells, which makes them applicable as a biological vector system to deliver genetic material to brain cells. There is a broad range of viral vectors available but the most commonly used are adeno-associated virus (AAV) vectors and lentivirus (LV) vectors. Important issues in viral gene delivery are stable transgene expression, limited immunogenicity, the induction of an inflammatory response, the (lack of) cell specific targeting efficiency, safety, toxicity, and the need for packaging cell lines [195].

Brain tropism (e.g., brain targeting) varies from virus to virus and has been shown not to be sufficient to pass the BBB following intravenous administration; therefore, the virus has to be injected directly into the brain. Moreover, various viral vectors have affinities for specific brain cells [190–196]. This could be improved if viral vectors could be produced with a homing/targeting device at their surface. Furthermore, the selectivity of gene transcription in the brain can be strongly enhanced by

application of brain selective promoters like the myelin basic protein (MBP) promoter [197], the neuron-specific enolase (NSE) promoter [198–200], the platelet-derived growth factor-β (PDGB-β) promoter [201, 202], the glial fibrillary acid protein (GFAP) promoter [203], and synthetic promoters like the CMV plus human growth hormone first intron enhancer and the CMV-chicken β-actin promoter [204, 205]. Moreover, the addition of a posttranscriptional regulatory element of the woodchuck hepatitis virus (WPRE) has enhanced the expression of GFP [206, 207]. Similarly, application of the replication origin (oriP) and Epstein–Barr nuclear antigen (EBNA-1) elements [208] can enhance episomal transcription of plasmids [209]. However, it has been indicated that application of such elements could induce tumorgeneity, which makes them not readily applicable in humans [210].

The Tet and Cre-Lox systems are other regulatory systems that have been applied, particularly for viruses that integrate their genetic material into the genome [211]. The Tet system can upregulate the expression of GFP in rat brain in the absence of doxycycline [212] while the Cre-Lox system allows a conditional but irreversible gene modification [213].

The *adeno-associated virus* (AAV) is a human parvovirus and contains a ssDNA. There are approximately 35 known serotypes. Several of them have been engineered into recombinant viral vectors from which the rAAV1, rAAV2, and rAAV5 are most widely used to deliver genes to the brain [193, 214–217]. In particular, these serotypes are most effective in the transduction of neurons [215, 218]. The attractive feature of the virus is that its replication is dependent on coinfection with a lytic helper virus, which precludes the reversion into a replication competent virus [219].

rAAV vectors are capsid virions composed of a nucleic acid genome surrounded by a proteinaceous shell. The composition of the capsid determines which cells they bind to and may enter. They enter the cell following binding to heparin sulfate proteoglycan (HSPG) receptors. Two other coreceptors seem to be helpful in this and finally the dsDNA enters the nucleus through the pore [220, 221].

Once in the cell, the rAAV genome is maintained as an extrachromosomal element or episomal element [190, 222–224], which eliminates the risk of proto-oncogene activation or endogenous gene inactivation, which may occur following insertion into the host genome [223]. Nevertheless, random integration of rAAV material does irregularly occur [190, 223, 225]. The episome might replicate along with the endogenous cellular DNA but can also be maintained as a nonreplicating extrachromosomal element [190]. In nondividing cells, the episome can be maintained as a stable element, resulting in long-term transgene expression in cells [190, 222–224, 226]. In addition, a safe and long-term correction of genetic diseases in animal models following a single administration has been demonstrated [191]. These viruses can be produced under current Good Manufacturing Practices (cGMPs) as outlined in the Code of Federal Regulations (21CFR) in the United States [191]. This all guarantees that pure and high-quality rAAV vectors can be produced for human use.

Expression of rAAV2 delivered vectors in animals has been shown for brain-derived neurotrophic factor (BDNF) in atrophy of spinal neurons [227]; and glial cell line mediated neurotrophic factor (GDNF), tyrosine hydroxylase (TH), aromatic L-amino acid decarboxylase (AADC), and guanosine triphosphate cyclohydrolase (GCH) in Parkinson disease [228–234]. In addition, rAAV vectors are

effective in the treatment of lysosomal storage diseases like mucopolysaccharidosis type I, II, and VII [235] and Niemann–Pick A [236]. In addition, it was demonstrated in mice that by targeting the deep cerebellar nuclei with AAV1 vectors capable of axonal transport, it was possible to get a widespread distribution throughout the cerebellum [236].

The major problem in delivering rAAV vectors to the brain is to get global brain transduction. This holds particularly for disorders caused by single-gene mutations such as lysosomal storage disorders and leukodystrophies, where a global brain transduction is desirable. Some vectors have been shown to have an enhanced neurotropism when expressing peptides that mimic binding domains for cytoplasmic dynein or NMDA receptors [237]. In addition, other viral vectors have been constructed that express capsid proteins that function as homing devices for targeted delivery of genes [238].

Another disadvantage is the occurrence of immune responses when animals have been treated with rAAV vectors, since this has resulted in killing of the transduced cells by cytotoxic T cells [190, 223, 225, 239, 240]. It was argued that this was caused by a slow uncoating of the rAAV in contrast to rapid uncoating [241, 242]. In addition, a humoral response can be elicited, which results in the production of antibodies. Moreover, memory cells are produced that will boost a strong humoral response when the same antigen is detected again. In this way, memory cells prevent the possibility of vector readministration [223].

Another feature is the packaging capacity of viruses. For rAAVs this is rather small (~4.5 kb) [190, 225, 243]. However, this may be increased up to 36 kb [244] by using (gutless) vectors, where as many viral genes as possible are deleted, only using those for the packaging sequence and sequences that define the beginning and end of the viral genome [190, 224, 240].

Lentivirus (LV) *vectors* are very promising vehicles to deliver therapeutic genes into the brain [190, 193, 194]. LV vectors are capsid virions surrounded by a lipid bilayer envelope [190]. LV vectors enter the cell following binding to HSPG; following membrane fusion, the virus moves along the microtubule by dynein-mediated transport. Finally, the dsDNA enters the nucleus through the pore [190]. LV vectors have a moderate packaging capacity with a maximum of 8 kb. LV vectors can be used to transduce a broad range of dividing as well as nondividing cells. In contrast to the rAAV vectors, they integrate into the host genome [196] and should therefore provide a longer and more stable transgene expression. However, they could also activate a proto-oncogene [190, 223, 224, 245] but since most CNS cells are terminally differentiated, the risk of tumorigenesis seems to be diminished [190, 225]. Nevertheless, the risk that vital genes are inactivated by random integration exists [190, 223, 245].

Furthermore, LV vectors seem to have a low immunogenicity. This seems to be caused by the fact that the genomes of these vectors do not encode any viral proteins. Indeed, low immunogenicity and prolonged transgene expression in the presence of preexisting lentiviral immunity was found, which should be encouraging for future use of these vectors in brain gene therapy [246].

Although viral delivery systems enter the cell by receptor-mediated endocytosis, this cell entry is not selective/specific since they can enter various cells, including brain cells, because of the lack of the expression of a selective/specific homing device. Therefore, rAAV and LV vectors can only effectively be delivered to the

brain by direct local injection/infusion. However, the diffusion from the site of administration in the brain is minimal; therefore, the region of cells that is transduced is restricted to a few millimeters from this site [190, 224]. To compensate for poor brain diffusion, usually the vector is administered by injections at multiple sites to increase the area of vector delivery. However, injection is associated with injury at the injection site, which could evoke a damaging response.

LV vectors have already been applied for gene therapy of various (brain) diseases like lysosomal storage diseases, Sandhoff disease and mucopolysaccharidosis I [247, 248], Parkinson disease [249, 250], ALS [251], epilepsy [252], prion disease [253], brain tumors [254], and the delivery of growth factors [250, 255].

In summary, one can say that today viral gene delivery to the brain can be efficient. Many applications have been demonstrated for brain gene therapy by rAAV and LV vectors, showing an increasing interest in the use of LV vectors. Critical points in this respect are global versus local delivery including the ability to reach the desired site of action (targeting efficiency). This may be improved considerably when homing/targeting devices are expressed at the outer surface of the virus particle [238], which will allow the vascular and global delivery of these systems to the CNS, thereby avoiding damage caused by direct injection/infusion. The selectivity of delivery can be further enhanced when tissue-selective/specific promoters are included in the vectors. rAAV vectors stay mainly episomal, while LV vectors integrate into the genome with the possibility to activate proto-oncogenes. Furthermore, immunological reactions may occur, but these seem to be reduced following application of LV vectors. In addition, rAAV and LV vectors have a rather small to moderate packaging capacity and can safely be produced under well controlled conditions.

With respect to invasive brain drug delivery strategies, one may conclude that these can be effective for local delivery (e.g., tumors) but not for the administration of drugs against more widespread diseases, including diffuse tumors and metastases. Therefore, since every neuron has its own capillary, the only route for an effective global delivery of biopharmaceutical drugs to the brain is the vascular route by application of nonviral systems. This is discussed further in the next section.

11.4.2 Global Brain Delivery by Vascular Nonviral Drug Administration

The advantage of the vascular route is the widespread transport by blood of the infused drug across the whole brain [256]. As has been mentioned, approximately each neuron has its own brain capillary for oxygen supply as well as the supply of other nutrients (see also Fig. 11.3A). This means that the vascular route is a very promising one for drug targeting and delivery to the whole brain. However, the main problem for drug transport to the brain is BBB passage.

Drug Transport Across the BBB There are various possibilities for drug transport across the BBB as shown in Fig. 11.4. Passive diffusion depends on lipophilicity and molecular weight [257]. Furthermore, the ability of a compound to form hydrogen bonds will limit its diffusion through the BBB [258]. In general, Lipinski's rule of five, as well as Abraham's equation, can be used to predict the passive transport of a drug molecule across the BBB [259–261]. Transport of hydrophilic compounds via the paracellular route is limited, while lipophilic drugs smaller than 400–600 Da may freely enter the brain via the transcellular route.

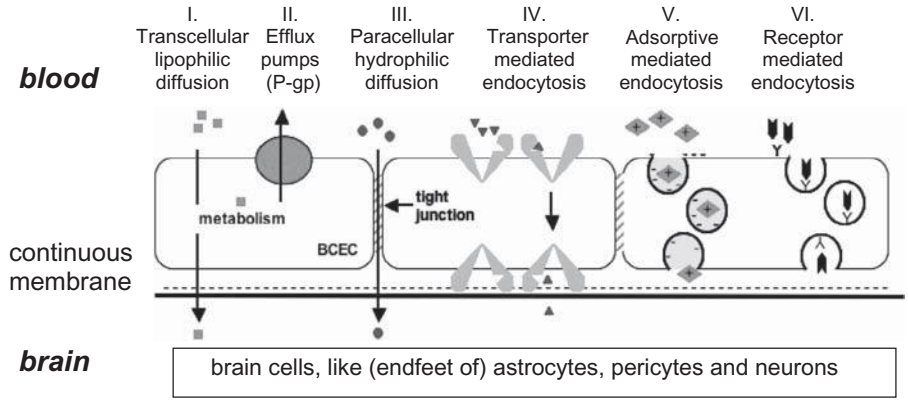


FIGURE 11.4 The various transport processes that may occur at the blood–brain barrier. (Adapted from Ref. 297.)

Active transport systems can be divided into absorptive-, carrier-, or receptor-mediated transcytosis. Absorptive-mediated transcytosis is initiated by the binding of polycationic substances (such as most cell-penetrating peptides) to negative charges on the plasma membrane [262, 263]. This process does not involve specific plasma membrane receptors. Upon binding of the cationic compound to the plasma membrane, endocytosis occurs, followed by the formation of endosomes. However, vesicular transport is actively downregulated in the BBB to protect the brain from nonspecific exposure to polycationic compounds. Therefore, forcing drugs to enter the brain by absorptive-mediated transcytosis goes against the neuroprotective barrier function, as demonstrated for anionic and cationic nanoparticles that disrupt the BBB [264].

Carrier-mediated transcytosis is used for the delivery of nutrients, such as glucose, amino acids, and purine bases, to the brain [265–267]. At least eight different nutrient transport systems have been identified, each transporting a group of nutrients of the same structure. Carrier-mediated transcytosis is substrate selective and the transport rate is dependent on the degree of occupation of the carrier [267, 268]. Therefore, only drugs that closely mimic the endogenous carrier substrates will be taken up and transported into the brain.

In contrast, receptor-mediated transcytosis enables larger molecules, such as peptides, proteins, and genes, to specifically enter the brain. Classical examples of receptors involved in receptor-mediated transcytosis are the insulin receptor [269], the transferrin receptor [270, 271], and the transporters for low density lipoprotein [272], leptin [273], and insulin-like growth factors [274].

Besides many influx mechanisms, several efflux mechanisms exist at the BBB as well. The best known is P-glycoprotein (P-gp) [33–37, 275, 276]. P-gp is a transmembrane protein, located at the apical membrane of the BCEC. It has a high affinity for a wide range of cationic and lipophilic compounds and therefore limits transport to the brain of many drugs including cytotoxic anticancer drugs, antibiotics, hormones, and HIV protease inhibitors [277]. Other multidrug resistance (MDR) efflux mechanisms at the BBB include the MDR related protein (MRP), such as MRP 1, 2, 5, and 6 [278]. In addition, as mentioned before (see Section 11.2.1), many other

transporters are present at the BBB, like the organic anion transporter (influx and efflux), the organic cation transport system (influx), and the nucleoside transporter system (influx) [36, 37].

In conclusion, research over the years has shown that the BBB is a dynamic organ, which combines restricted diffusion to the brain for endogenous and exogenous compounds with specialized transport mechanisms for essential nutrients.

Global Brain Delivery by Enhanced Passive Vascular Drug Delivery Large molecules (e.g., drug conjugates) or drug delivery systems (nanosized carrier systems including polymers, emulsions, micelles, liposomes, and nanoparticles) may reach the brain by passive targeting similar to small molecules, meaning that their distribution is mainly determined by physicochemical and physiological conditions [279–281]. Mostly this results in a very poor distribution into the brain. However, enhanced distribution may be obtained when the permeability of blood vessels in the target tissue has been increased due to disease conditions such as in tumors. Such "enhanced permeability and retention" has been observed in many cases [282, 283], showing increased accumulation of drug in those (mostly peripheral) areas. Unfortunately, drug delivery to the brain by enhanced permeability and retention is not effective in treating human brain diseases.

Enhanced drug delivery to the brain has been achieved in various other ways also. One is the osmotic disruption/shrinking of the BBB by intracarotid administration of a hypertonic mannitol solution. By subsequent administration of drug, substantial increased concentrations can be achieved in brain or tumor tissue. However, because the BBB is temporarily opened under such conditions, which has to be done under anesthesia, it has the disadvantage also that neurons may be damaged permanently due to unwanted blood components entering the brain [284]. Therefore, this procedure is only applicable in life-threatening brain diseases (e.g., tumors) [186].

A less "aggressive" approach seems to be the intracarotid administration of alkylglycerol [285], which enhances mainly drug transport by the paracellular route. In rats with brain implanted C_6-glioma, increased tissue fluorescence was found in both tumor tissue and brain surrounding the tumor following coadministration of FITC-dextran (40 kDa). Similar data on brain tissue were observed following coadministration of methotrexate to nude mice. However, anesthesia is needed to perform this type of administration, which limits its applicability to life-threatening brain diseases like brain tumors. In addition, no human data are available yet.

Another procedure is the application of a bradykinin-analogue (RMP-7) that has been shown to increase BBB permeability by opening of the tight junctions via a receptor-mediated mechanism (Ceroport®), which resulted in an enhanced uptake of carboplatin in C_6-glioma implanted in rat brain [286]. However, a Phase II study could not show improved carboplatin efficacy in combination with RMP-7 [287]. In addition, a Phase III study with RMP-7 and carboplatin with radiotherapy was stopped because of no increased efficacy.

At last, a general aspect about these agents is the possibility that besides opening of the BBB and the associated brain disturbances, application in the treatment of brain tumors could also cause an enhanced incidence of tumor dissemination via vascular spread of tumor cells [288].

Enhanced delivery can be achieved also by application of so-called protein transduction domains (PTDs) (for reviews see Refs. 289 and 290). These PTDs are typical

amino acid sequences that are capable of enhancing delivery of large molecules into cells. Typical examples are the Tat-PTD [291], the homeodomain of Antennapedia [292], SynB-vectors [293], and others [294, 295]. These peptides are basic molecules (and therefore cations) that are able to enhance protein uptake by cells mainly by increased adsorptive-mediated endocytosis. Toxicity profiles of the various PTDs are an important issue [296] but because this approach lacks cell or tissue selectivity it seems to be particularly suitable for local drug delivery. Clinical proof of principle has recently been demonstrated with SynB-vectors to enhance the transport of morphine-6-glucuronide to the brain (www.syntem.com).

Recently, therapeutic gene silencing of apolipoprotein B has been demonstrated in mice following administration of a high dose (50 mg/kg) of modified siRNAs [297]. This was achieved by coupling cholesterol to siRNA. Increased uptake by liver, heart, kidney, adipose, and lung tissue was seen but not by brain. The uptake into these tissues was mainly established by lipophilization of the siRNA by cholesterol and the resulting increase in plasma half-life from 6 min (naked siRNA) to 95 min (chol-siRNA).

A similar approach has been applied by coupling lipophilic and amphiphilic groups (stearoyl groups or Pluronic block copolymers) to polypeptides to enhance the uptake of these compounds by the brain. However, no selectivity in brain uptake has been demonstrated by applying such modifications [298].

In conclusion, all these approaches are able to enhance more or less the uptake into various tissues but lack selective/specific homing devices that are needed to target drugs to the brain and to selectively/specifically increase drug delivery to the brain.

Global Brain Delivery by Active Vascular Receptor-Mediated (Nonviral) Drug Targeting Active physiological or disease-induced drug targeting strategies involve the application of a homing device or technology to apply endogenous transport mechanisms for site selective/specific delivery in the body [273, 275, 299]. With respect to the BBB, this particularly involves receptor-mediated transcytosis systems at the BBB to reach extracellular or intracellular targets in the brain. The advantage of active targeting is the increase of the amount of drug in the target tissue, thereby increasing the pharmacological response and reducing (peripheral) side effects. However, when the homing device is not specific, side effects can still occur. Interestingly, targeting efficiency can be increased when the target is disease-induced such as occurs for the diphtheria toxin receptor under inflammatory disease conditions (see following paragraphs). The focus of the following paragraphs is on active targeting by receptor-mediated transcytosis, and several examples of receptor-mediated transcytosis that have successfully been employed to target drugs to the brain are reviewed.

In general, receptor-mediated transcytosis occurs in three steps: receptor-mediated endocytosis of the compound at the luminal (blood) side, movement through the endothelial cytoplasm, and exocytosis of the drug at the abluminal (brain) side of the brain capillary endothelium [12]. Upon receptor–ligand internalization, chlathrin-coated vesicles are formed, which are approximately 120 nm in diameter [300, 301]. These vesicles may transport their content to the other side of the cell or go into a route leading to (protein) degradation. Indeed, at least two important routes for degrading proteins have been identified including the lysosomal and the

ubiquitin-proteasome route [302–304]. Nevertheless, protein delivery to the brain has been shown to be effective. Therefore, receptor-mediated transcytosis allows the specific delivery/targeting of larger drug molecules or drug-carrying particles (like liposomes or nanoparticles) to the brain.

Transferrin Receptor The most widely characterized receptor-mediated transcytosis system for the targeting of drugs to the brain is the transferrin receptor (TfR). TfR is a transmembrane glycoprotein consisting of two 90 kDa subunits. A disulfide bridge links these subunits and each subunit can bind one transferrin molecule [305]. The TfR is expressed mainly on hepatocytes, erythrocytes, intestinal cells, and monocytes, as well as on endothelial cells of the BBB [306, 307]. Furthermore, in the brain the TfR is expressed on choroid plexus epithelial cells and neurons [305]. The TfR mediates cellular uptake of iron bound to transferrin.

Drug targeting to the TfR can be achieved by either using the endogenous ligand, transferring, or using an antibody directed against the TfR (OX-26 anti-rat TfR). Each of these targeting vectors has its advantages and disadvantages. For transferrin, the *in vivo* application is limited due to high endogenous concentrations of transferrin in plasma and the likely overdosing with iron when one tries to displace the endogenous transferrin with exogenously applied transferrin-containing systems. However, recent studies in our group [309] have shown that liposomes tagged with transferrin are suitable for drug delivery to BBB endothelial cells *in vitro*, even in the presence of serum. OX-26 does not bind to the transferrin binding site and is therefore not displaced by endogenous transferrin.

The TfR is responsible for iron transport to the brain. So far, the intracellular trafficking of transferrin and OX-26 upon internalization via the TfR has not yet been elucidated. Some literature reports suggest transcytosis of transferrin across the BCECs, while others claim endocytosis of transferrin, followed by an intracellular release of iron and a subsequent return of apo-transferrin to the apical side of the BCEC [268, 309, 310]. Moos and Morgan [305] have shown that the transcytosis of iron exceeds the transcytosis of transferrin across the BBB, supporting the second theory. Furthermore, these authors have proposed a new theory in which the TfR–transferrin complex is transcytosed to the basolateral side of the BCEC, where transferrin remains bound to the TfR, but iron is released into the brain extracellular fluid [311]. Subsequently apo-transferrin bound to the TfR will recycle back to the apical side of the BBB. This theory is supported by data from Zhang and Pardridge [309], who found a 3.5-fold faster efflux from brain to blood of apo-transferrin than holo-transferrin. In addition, in a recent publication Deane et al. [312] illustrated that free iron is rapidly taken up by brain capillaries and subsequently released into the brain extracellular fluid and CSF at controlled moderate to slow rates.

The mechanism of transcytosis of OX-26 is not yet fully elucidated either. Pardridge and colleagues have shown efficient drug targeting and delivery to the brain *in vivo* by applying OX-26 (examples can be found in [265, 313–315]. In contrast, Broadwell et al. [316] have shown that both transferrin and OX-26 are able to cross the BBB, but that the transcytosis of transferrin is more efficient. Furthermore, Moos and Morgan [317] have shown that OX-26 mainly accumulates in the BCECs and not in the postcapillary compartment. In addition, iron deficiency did not increase OX-26 uptake in rats. Our data as well as literature reports show that iron deficiency causes an increase in TfR expression [306, 318, 319]. Therefore, it is

expected that the uptake of OX-26 would increase as well. The data by Moos and Morgan [317] suggest that OX-26 transcytosis might result from a high-affinity accumulation by the BCECs, followed by a nonspecific exocytosis at the basolateral side of the BCECs. In addition, these authors found a periventricular localization of OX-26, which suggests that OX-26 probably also is transported across the BCSFB.

Although the mechanism of transcytosis of transferrin and OX-26 may not yet be fully elucidated, it is important to realize that drug delivery to the brain via the TfR is efficient. By these means, vasoactive intestinal peptide (VIP), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and peptide nucleic acids, as well as pegylated immunoliposomes containing plasmid DNA encoding for β-galactosidase, tyrosine hydroxylase, and short hairpin RNAs, have all been made available to the brain [320]. However, OX-26 is an antibody against the rat TfR and does not bind to the human TfR, making it impossible to translate this technology to the clinic. Moreover, rat antibodies will cause immunogenic reactions in humans, unless they are humanized. The preparation of humanized or chimeric antibodies is difficult and in some cases this may lead to a loss of affinity for the target receptor [321]. In addition, one can argue that the administration of antibodies directed against such an important uptake mechanism involved in iron homeostasis poses a risk for human application.

Still, preferably, a targeting vector directed to the TfR would be small and nonimmunogenic and should initialize internalization of the TfR upon binding. Xu et al. [322] have used a single-chain antibody Fv fragment against the human TfR, which was tagged with a lipid anchor for insertion into a liposomal bilayer [323]. The molecular weight of this antibody fragment, including the lipid anchor, was approximately 30 kDa. In addition, Lee et al. [323] have used a phage-display technique to find small peptide ligands for the human TfR. They obtained 7 mer and 12 mer peptides that bind to a different binding site than transferrin and are internalized by the TfR. Although these small peptides can also exert immunogenic reactions in humans, they are promising ligands for drug targeting to the human TfR on the BBB.

Insulin Receptor Another widely characterized, classical, receptor-mediated transcytosis system for the targeting of drugs to the brain is the insulin receptor. Again, just as for the TfR system, Pardridge and colleagues have documented use of the insulin receptor for the targeted delivery of drugs to the brain.

The insulin receptor is a large 300 kDa protein and is a heterotetramer of two extracellular α and two transmembrane β subunits. Each β chain contains a tyrosine kinase activity in its cytosolic extension. The α and β subunits are coded by a single gene and are joined by disulfide bonds to form a cylinder structure. Primarily, insulin binds and changes shape of the receptor to form a tunnel to allow entry of molecules such as glucose into the cells. The insulin receptor is a tyrosine kinase receptor and induces a complex cellular response by phosphorylating proteins on their tyrosine residues. The binding of a single insulin molecule into a pocket created by the two α chains effects a conformational change in the insulin receptor, so that the β chains approximate one another, and carries out transphosphorylation on tyrosine residues. This autophosphorylation is necessary for the receptor to internalize into

endosomes. The endosomal system has been shown to be a site where insulin signaling is regulated, but also the degradation of endosomal insulin occurs there. Most of the insulin is degraded, but less so in endothelial cells [324], whereas the receptors are largely recycled to the cell surface. Endocytosis is not necessary for insulin action but probably is important for removing the insulin from the cell, so the target cell for insulin responds in a time-limited fashion to the hormone. This endocytosis mechanism of the insulin receptor has been exploited for the targeting of drugs to the brain.

As for transferrin, the *in vivo* application of insulin as the carrier protein is limited, mainly due to the high concentrations of insulin needed and the resulting lethal overdosing with insulin. Therefore, drug or gene delivery, for instance, to rhesus monkeys is performed with the murine 83–14 MAb that binds to the exofacial epitope on the α subunit of the human insulin receptor. The MAb has a BBB permeability surface area (PS) product in the primate that is ninefold greater than murine MAbs to the human TfR [325]. By using this MAb, Pardridge and co-workers have successfully made available to the brain of primates radiolabeled amyloid-β peptide$_{1\text{-}40}$ (A$\beta_{1\text{-}40}$), serving as a diagnostic probe for Alzheimer disease, and pegylated immunoliposomes containing plasmid DNA encoding for β-galactosidase [325].

Unfortunately, the 83-14 MAb cannot be used in humans owing to immunogenic reactions to this mouse protein. However, genetically engineered, effective, forms of the MAb have now been produced, which may allow for drug and gene delivery to the human brain [326]. Still, one can argue that the administration of antibodies directed against such an important mechanism involved in glucose homeostasis poses a risk for human application.

LRP1 and LRP2 Receptors During the past few years, the LRP1 and LRP2 (also known as megalin or glycoprotein 330) receptors have been exploited to target drugs to the brain in a similar fashion as the transferrin and insulin receptors. Both LRP1 and LRP2 receptors belong to the structurally closely related cell surface LDL receptor gene family. Both receptors are multifunctional, mutiligand scavenger and signaling receptors. A large number of substrates are shared between the two receptors, like lipoprotein lipase (LPL), α_2-macroglobulin (α_2M), receptor-associated protein (RAP), lactoferrin, tissue- and urokinase-type plasminogen activator (tPA/uPA), plasminogen activator inhibitor (PAI-1), and tPA/Upa:PAI-1 complexes. More specific ligands for the LRP1 receptor are melanotransferrin (or P97), thrombospondin 1 and 2, hepatic lipase, factor VIIa / tissue-factor pathway inhibitor (TFPI), factor VIIIa, factor IXa, A$\beta_{1\text{-}40}$, amyloid-β precursor protein (APP), C1 inhibitor, complement C3, apolipoprotein E (apo E), *Pseudomonas* exotoxin A, HIV-1 Tat protein, rhinovirus, matrix metalloproteinase 9 (MMP-9), MMP-13 (collagenase-3), sphingolipid activator protein (SAP), pregnancy zone protein, antithrombin III, heparin cofactor II, α_1-antitrypsin, heat shock protein 96 (HSP-96), and platelet-derived growth factor (PDGF, mainly involved in signaling) [327–331], where apolipoprotein J (apo J, or clusterin), Aβ bound to apo J and apo E, aprotinin, and very low density lipoprotein (VLDL) are more specific for the LRP2 receptor [332, 333].

The group of Richard Béliveau first reported that melanotransferrin/P97 was actively transcytosed across the BBB and suggested that this was mediated by the

LRP1 receptor [331]. Melanotransferrin is a membrane-bound transferrin homolog that can also exist in a soluble form and is highly expressed on melanoma cells compared to normal melanocytes. Intravenously applied melanotransferrin delivers the majority of its bound iron to the liver and kidney, where only a small part was taken up by the brain [334]. After conjugation to melanotransferrin, the group of Béliveau was able to successfully deliver doxorubicin to brain tumors in animal studies [335, 336]. This melanotransferrin-mediated drug targeting technology (now designated NeuroTrans™) is currently further developed by BioMarin Pharmaceuticals Inc. (Novato, CA, USA) for the delivery of enzyme replacement therapies to the brain. Interestingly, together with researchers from BioMarin, Pan et al. [337] recently reported on the efficient transfer of RAP across the BBB by means of the LRP1/LRP2 receptors, suggesting a novel means of protein-based drug delivery to the brain. RAP is a 39 kDa protein that functions as a specialized endoplasmic reticulum chaperone assisting in the folding and trafficking of members of the LDL receptor family. In yet unpublished results, the group of Béliveau have now filed a patent application on the use of the LRP2-specific ligand aprotinin, and more specifically on functional derivatives thereof (e.g., angio-pep1), thereby providing a noninvasive and flexible method and a carrier for transporting a compound or drug across the BBB [338]. Aprotinin (Trasylol®) is known as a potent inhibitor of serine proteases such as trypsin, plasmin, and tissue and plasma kallikrein and is the only pharmacologic treatment approved by the U.S. Food and Drug Administration to reduce blood transfusion in coronary artery bypass grafting [339].

In addition to being a tumor marker protein, melanotransferrin is also associated with brain lesions in Alzheimer disease and is a potential marker of the disorder [340]. In addition, the proposed receptor for melanotransferrin, LRP1, has genetically been linked to Alzheimer disease and may influence APP processing and metabolism and Aβ uptake by neurons through α_2M (α_2M is one of the Aβ carrier proteins next to, e.g., apo E, apo J, transthyretin, and albumin) [332, 341]. Furthermore, a close relationship with RAGE (receptor for advanced glycation end products) in shuttling Aβ across the BBB has been described [332, 341]. In addition, the LRP2 receptor has also been described to mediate the uptake of Aβ complexed to apo J and apo E across the BBB [333, 342, 343]. This complex interaction with Alzheimer disease makes the safety of the use of the LRP1/LRP2 receptors for the targeting of drugs to the brain difficult to predict in human application; especially when one considers that the complex signaling function of these receptors is included in the assessment (e.g., the control of permeability of the BBB, vascular tone, and the expression of MMPs [327]), as well as the fact that both receptors are critically involved in the coagulation-fibrinolysis system. On top of that, melanotransferrin was also reported to be directly involved in the activation of plasminogen [344], and high plasma concentrations of melanotransferrin are needed to deliver drugs to the brain, resulting perhaps in dose limitations because of the high iron load in the body.

The same line of reasoning for the interactions at the level of the uptake receptors may apply to the use of RAP and aprotinin (derivatives). On the other hand, the latter has already been successfully applied to humans usually without severe side effects, indeed making the peptide derivatives potentially safe drug carriers. As for RAP, though, no results on the efficacy or capacity of the aprotinin peptides as carrier for drugs have been made available yet.

Diphtheria Toxin Receptor Recently, our group has identified a novel human applicable carrier protein (known as CRM197) for the targeted delivery of conjugated proteins across the BBB [345, 346]. Uniquely, CRM197 has already been used as a safe and effective carrier protein in human vaccines for a long time [347] and recently also as a systemically active therapeutic protein in anticancer trials [348]. This has resulted in a large body of prior knowledge on the carrier protein, including its transport receptor and mechanism of action, receptor binding domain, conjugation and manufacturing process, and kinetic and safety profile both in animals and humans. CRM197 delivers drugs across the BBB by the well characterized, safe, and effective mechanism called receptor-mediated transcytosis. From the literature, it was already known that CRM197 uses the membrane-bound precursor of heparin-binding epidermal growth factor-like growth factor (HB-EGF) as its transport receptor [349]. This precursor is also known as the diphtheria toxin receptor (DTR). In fact, CRM197 is a nontoxic mutant of diphtheria toxin. Membrane-bound HB-EGF is constitutively expressed on the BBB, neurons, and glial cells [350]. Moreover, HB-EGF expression is strongly upregulated on cerebral blood vessels by ischemic stroke and in gliomas [351, 352], which may lead to a site-selective improvement of the therapeutic efficacy of the targeted drugs in the brain.

By means of a dynamic cell culture model of the BBB, our group was able to demonstrate the functional expression of the DTR, safety of the CRM197 carrier protein, and specific transport efficacy of CRM197 carrier protein conjugated to a 40 kDa enzyme (horseradish peroxidase, HRP, serving as a "model" protein drug) and DTR-targeted pegylated liposomes containing HRP. In addition, the *in vivo* proof-of-principle with this novel brain drug targeting technology was demonstrated by the specific brain uptake of DTR-targeted HRP in guinea pigs [345, 346].

Although HB-EGF is expressed in species including human, monkey, rat, and mouse with a similar tissue distribution, only rats and mice are resistant to diphtheria toxin because of an amino acid substitution in the receptor-binding domain on HB-EGF that reduces binding of diphtheria toxin to rodent HB-EGF [346, 353]. Fortunately, a transgenic mouse (conditionally) expressing the human DTR was recently generated by Cha and co-workers [354], allowing researchers to specifically study the brain drug delivery technology in mice as well.

Another known complication of the bacterial CRM197 protein is that neutralizing antibodies against diphtheria toxin may develop or may already be present in serum of the recipient because of earlier vaccinations, thereby reducing the efficacy of the drug delivery system. There are, however, several lines of evidence that such an immune response to CRM197 can occur, but, most importantly, that it is not really a problem in the clinic, at least not for the treatment of acute indications. In fact, the clinical studies performed by Buzzi et al. [348] indicate that preexisting levels of neutralizing antibodies were rather decreased 30 days after repeated treatment with CRM197.

Another interesting aspect of the DTR is the fact that this receptor is strongly upregulated under inflammatory disease conditions [346] such as occur in many brain diseases like Alzheimer disease, Parkinson disease, multiple sclerosis, ischemia, encephalitis, epilepsy, and tumors [351]. On the one hand, such a situation may enhance the therapeutic effect by disease-induced targeting. On the other hand, this may be used to image (inflammatory) disease areas in the brain. For instance, it has

already been shown that the DTR is upregulated in the brain following seizures [355]. By applying CRM197-coated liposomes loaded with an MRI-enhancing agent (e.g., gadolinium), it would be possible to image such disease areas, as has been done for the glucose receptor [356]. In particular, this would be helpful to investigate the extent of damage/recovery and disease progression/therapy in time of many brain diseases, particularly epilepsy, ischemia, and brain tumors.

11.5 CONCLUSION

There is still an unmet need for the treatment of brain diseases. In addition to the presently applied small molecular drugs, there are many good biopharmaceutical drugs that, unfortunately, cannot enter the brain in sufficient quantities to be effective. Therefore, (new) technologies have to be applied and developed to accomplish this. In this chapter we have tried to give insight into the present *in vitro* [357] and *in vivo* [358] techniques to study and improve drug absorption and distribution of biopharmaceutical drugs (e.g., proteins, RNAi, and genes) to the brain.

In vitro techniques to study BBB drug transport and BBB functionality should use an endothelial/astrocyte coculture system for establishing a tight and functional BBB system. Some systems with BBB barrier enhancers (e.g., cAMP, phosphodiesterase inhibitors, glucocorticoids) could be used to estimate BBB drug transport but may lack BBB functionality. From the *in vivo* methods, the noninvasive MRI techniques seem to be very promising and impressive advancements have been made to image (diseased) brain by application of MRI contrast-enhancing agents. The development of technologies to actively target such agents to the (diseased) brain will open a wide area for diagnostic investigations and the possibility to monitor disease progression in the brain.

With respect to the therapeutic treatment of brain diseases, the only human applied technology to deliver (biopharmaceutical) drugs to the brain is by viral delivery. Up to now viral delivery to the brain has to be performed by direct injection/infusion into the brain because of insufficient brain targeting due to the lack of a homing device, which makes it only sufficient for local delivery. Therefore, the most promising technology in our opinion is active vascular nonviral receptor-mediated targeting. Vascular nonviral active targeting has been shown to be effective and selective for global delivery of drugs to the brain in several animals. By using antibodies against receptors (e.g., transferrin and insulin receptor), it has been demonstrated that proteins (enzymes), RNAi, and genes could be targeted to the brain. However, these applications cannot be transferred directly to the human situation: first, because the antibodies used in animals cannot (without humanization) directly be applied in humans; and second, antibodies are meant to stick to their counterpart, which means that administration of these will reduce the availability of receptors at the BBB and in the brain. Moreover, several of these receptors (insulin receptor and the multiligand LRP1 and LRP2 receptors) are involved in signaling too [27, 359]. Therefore, brain targeting by such antibodies may interfere with both the transport of endogenous ligands (e.g., insulin, transferrin) and brain signaling and cause downregulation of receptors; and therefore these antibodies should not be used for chronic administration. Furthermore, the melanotransferrin receptor seems to be involved in the release of plasmin [344] and therefore with the

blood clotting cascade, while the LRP1 receptor seems to be involved with apolipoprotein J transport in a complex with Alzheimer's amyloid-β [343]. Interference with such transport systems poses a potential hazard. In this respect, the DTR seems to be a human applicable, safe, and effective uptake receptor for the targeting of drugs to the brain. Moreover, the DTR does not have an endogenous ligand, so there is no competition for transport. Furthermore, CRM197 is already safely applied to humans (illustrating that binding to the DTR *per se* does not result in serious side effects), where other carrier systems involve potential safety hazards in human application. However, even though specific brain uptake of DTR-targeted enzyme was already established in guinea pigs, the technology now awaits further *in vivo* validation in terms of kinetics of brain distribution and efficacy of targeted drugs in relevant disease models of the CNS.

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12

TRANSPORTER INTERACTIONS IN THE ADME PATHWAY OF DRUGS

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12.1 INTRODUCTION

Approximately 30% of the human genome encodes membrane proteins [1]. It has been estimated that as many as 1200 of these genes code for potential drug transporters [2]. Given the number of transport proteins expressed on cell membranes,

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it stands to reason that interactions of therapeutic agents with endogenous transporter systems could have a major impact on the absorption, distribution, metabolism, and excretion (ADME) properties of drugs. Transporter interactions can impact on the ADME pathway in two distinct and important ways. First, if a drug is a substrate for a specific transporter involved in absorption, distribution, or elimination, the transporter can influence the ADME properties of the drug itself. Examples of this include increased intestinal absorption of cephalosporins through transport by a peptide transporter in the intestine [3, 4], and decreased intestinal absorption of taxol by the efflux transporter, P-glycoprotein [5, 6]. Second, a drug may act as either an inhibitor or inducer of a selective transporter involved in the ADME properties, thereby altering the ADME properties of another drug. In this case, the result would be a drug–drug interaction that changes the pharmacokinetics of the drug. Athough such interactions were in the past considered to be entirely drug metabolism (cytochrome P450-mediated) related, it is now recognized that transporters may also be involved [7, 8].

Identification of the transport properties of compounds during the drug discovery process is essential for optimization of the ADME properties of therapeutic agents. In this regard, the pharmaceutical scientist has a variety of tools with which to probe transporter interactions of compounds. These include cell-based uptake, absorption and transport assays, *in situ* models that can isolate the pertinent absorption, distribution, or elimination tissues, and computational models that can simulate and/or predict transporter interactions. In addition, transgenic mouse models have made it possible to examine the impact of selected transport systems on the ADME process at the whole animal level. This chapter identifies some of the more important transporters that can influence the ADME properties of drugs. In addition, a strategy for evaluating compounds for transporter interactions is discussed that incorporates *in silico*-based modeling and *in vitro* and *in vivo* transporter studies.

12.2 TRANSPORT SYSTEMS IMPACTING DRUG ADME

There are several types of transport systems involved in moving solutes and macromolecules across cellular barriers. These include primary active transport systems that rely directly on ATP hydrolysis to move solutes across the membrane. Examples include the drug efflux transporters such as P-glycoprotein (P-gp), multidrug resistance associated protein (MRP), and breast cancer resistant protein (BCRP). Transporters in this group belong to the ATP binding cassette (ABC) superfamily of proteins [9–11]. In general, these transporters consist of 6–17 membrane spanning domains, one or more nucleotide binding domains, and one or more drug binding domains [10–12]. As these transporters rely directly on ATP hydrolysis, they have the capability of transporting the solutes and macromolecules against a concentration gradient. In mammalian cells, the direction of transport is usually directed toward removing compounds from the cell (i.e., outwardly directed).

Drug efflux transporters have been closely linked with the development of drug resistance in cancer [9, 10], bacterial [13], and parasitic [14] cells. These same drug efflux transporters are also present in normal tissue such as epithelial cells of the intestine, liver, and kidney, and endothelial cells forming the blood–brain barrier [15–18]. While the drug efflux transporters serve an important protective function,

preventing the absorption and distribution and speeding the elimination of potentially toxic xenobiotics, due to their rather promiscuous nature (i.e., ability to transport a wide variety of chemical compositions), they also have a substantial impact on the ADME properties of drugs. P-glycoprotein, the most widely studied drug efflux transporter, contributes to the low oral bioavailability of several drugs [19]. Furthermore, even those drugs that can overcome P-gp efflux in the intestine are still subject to restricted tissue distribution and increased clearance [20]. For these reasons, much effort has been placed on the identification of potential drug efflux transporter interactions early in the drug development process.

Another type of transport system present in the cell is secondary active transporters. In contrast to primary active transporters that rely on ATP hydrolysis, the secondary active transporters utilize electrochemical gradients to supply the energy required to move the solutes into or out of the cell. Thus, the "energy" for these transporters comes from the movement of ions, typically either sodium or hydrogen, down their concentration gradient. Examples of secondary active transporters include the sodium-dependent amino acid transporters found in the intestine and blood–brain barrier [21, 22], and the hydrogen ion-dependent peptide transporters (PepT1 and PepT2) found in the intestine, kidney, lung, and blood–cerebrospinal fluid barrier [23–25].

From a drug transport perspective, PepT1 and PepT2 deserve special mention. Both of these transporters were the first mammalian transporters identified that use the electrochemical proton gradient as a driving force [26]. The "preferred" substrates for PepT1 and PepT2 are di- and tripeptides [27]. This property of PepT transporters has been exploited to aid in the absorption of peptide and peptidomimetic agents in the intestine and lung [28, 29]. An example of this is the angiotensin converting enzyme (ACE) inhibitors [28]. However, unlike many of the solute and nutrient transporters that have relatively rigid structural requirements for transport substrates, the PepT transporters accommodate a wide variety of compounds. Consequently, not only are PepT1 and PepT2 able to aid in the intestinal absorption (PepT1) and kidney reabsorption (PepT2) of peptidomimetic drugs [28], but they can also influence the ADME properties of antibiotics such as cephalosporin [3, 4], and nucleoside-based antiviral prodrugs such as valacyclovir [30]. Although PepT1 and PepT2 share the same set of substrates, they differ in their affinity and capacity. Whereas PepT1 is a low affinity, high capacity transporter, PepT2 has high affinity and low capacity for the same substrates [23]. In addition, the presence of PepT2 on pulmonary epithelial cells [24, 29, 31] and epithelial cells of the choroid plexus, which form the blood–cerebrospinal fluid barrier [25], suggests this transporter could also influence drug distribution of selected therapeutic agents in these tissues.

A third type of transporter class is the solute carriers that allow the passage of compounds across the cellular barrier through a process involving facilitated diffusion [32]. In facilitated diffusion, the transported molecule interacts with the protein carrier, and through conformational changes in the protein, the molecule is allowed to pass through the lipid membrane. Cellular uptake of many nutrients such as glucose [33], various nucleotides [34, 35], and creatine [36] are known to occur through facilitated diffusion processes. These carriers display the same characteristics of saturability and solute selectivity as observed with the active transporters. However, unlike primary or secondary active transport systems, the carriers involved

in facilitated diffusion processes are not sensitive to metabolic inhibitors. In addition, the carriers involved in facilitated diffusion are bidirectional in that the solute will follow a concentration gradient whether that is directed into or out of the cell. While such carriers are important for nutrient absorption and distribution, the impact of facilitated diffusion on ADME properties of drugs is minimal, due in large part to the stringent structural requirements for these transporters.

12.3 COMPUTATIONAL MODELING OF TRANSPORTER INTERACTIONS

A number of criteria have to be met to successfully develop a drug-like compound. It has to be absorbed into the bloodstream and be distributed and bound to targeted tissues. Furthermore, in order to avoid toxic effects, many drugs require biotransformation for efficient elimination from the body. All of these aspects are significantly mediated or influenced, at some level, by transporter proteins. This has made the identification of transporter interactions a very important part of the ADME process. The following section summarizes the various options available for examining transport-mediated influences in the ADME pathway.

Prediction of the passive permeability of a compound using computational modeling is relatively straightforward and is aided by a very large training set of compounds with established physicochemical properties (i.e., lipophilicity, H-bonding potential, solubility, and stability) with which to generate the computer simulations. However, prediction of permeability when transporter interactions are involved is much more difficult. Indeed, when correlating actual permeability as accessed in either cell based or *in vivo* systems, to that predicted by computer modeling, it is often the outliers that have either metabolism or transport issues influencing permeability. Despite the added complexity, computational modeling of transporter interactions is an area of intense interest. Numerous efforts have been made to characterize various transporter/carrier proteins, including the PepT1, and the drug efflux pumps such as P-gp and MRP. However, little is known about these proteins at the molecular level due to the limited availability of high resolution crystal structures. Determination of crystal structure for transport proteins is important not only for understanding the function of these transporters but also for defining the structure–activity properties required for compounds transported by specific systems. The two general approaches used to model drug transporter systems include transporter-based modeling and substrate-based modeling [37]. Each of these approaches to computer modeling of transporter interactions is described.

12.3.1 Transporter-Based Modeling

The transporter-based approach focuses on the three-dimensional structure of the transport protein to assist in understanding the drug transport process. This approach uses the known or predicted three-dimensional structure of the protein to map out the topography of the transporter in the cell membrane. As would be predicted, the better the information is concerning the three-dimensional structure of the transporter being examined, the better the resulting computational model will be. However, complete knowledge of the three-dimensional structure is not essential

as a transporter-based approach can be applied when the target transporter shares the same number of transmembrane domains (TMDs) and an adequate level (generally >30%) of amino acid sequence identity with a second transporter, that has a known crystal structure available. This approach can be used even when the two transporters are not functionally related. For example, modeling of the apical sodium-dependent bile acid transporter (ASBT) was based on the crystal structure of bacteriorhodopsin [38]. In addition, the three-dimensional structure of the human facilitative glucose transporter (GLUT1) was predicted by using the crystal structure of a bacterial transporter, glycerol-3-phosphate transporter (GlpT), as a template [39]. Furthermore, important insights regarding the functionality of a target transporter can still be generated by modeling partial domains of the transporter when there is an absence of appropriate templates for a full-length transporter [37]. In studying the sulfate transporter, Rouached et al. [40] avoided modeling the transmembrane domains in their entirety because of the lack of appropriate templates. However, they identified the bacterial SpoIIAA protein as a template for the functionally unknown C-terminal sulfate transporter and anti-sigma antagonist (STAS) domain and directly generated a three-dimensional model for this domain [40].

In the transporter-based approach, the structure of a target transporter can be calculated using homology modeling techniques, including several commercially available programs, such as Modeller [41], InsightII homology [42], and ICM (MolSoft, San Diego, CA) [37]. Among these, Modeller is the most widely applied program. It follows four general steps, including template selection, sequence alignment, model generation, and model validation. These four steps are repeated as a cycle until no further improvements to the model are observed. Among the four steps, validation is an essential process to ensure accuracy of the generated model. Modeller generates several models based on the sequence alignment, which are, in turn, evaluated by internal self-consistency checks and external programs that verify structural protein quality. Examples of external programs used to verify protein structures are Procheck [43] and WHAT IF [44]. Generally, these programs develop rules and parameters extracted from available crystal structures and apply these to the model structure. The fitness that is represented by a "fit score" indicates the compatibility of the model to the available crystal structures [37]. The transporter-based modeling approach now enables the elucidation of three-dimensional structures for a large number of transporters that would otherwise not be available to scientists. Examples include the glucose transporter (GLUT1) [39], P-gp [45], and MRP1 [46].

Both P-gp and MRP1 belong to the ABC superfamily of transporters that have been extensively studied. The initial models of both P-gp and MRP are based on the crystal structures of bacterial transporters. P-gp was initially modeled using the crystal structure of the *Escherichia coli* lipid A transporter (MsbA), which shares ~30% homology with P-gp [45]. Subsequently, Stenham and colleagues [47] constructed an atomic detail model for P-gp based on assessment of the crystal structure of MsbA in relation to their disulfide cross-linking data. The two diverging models of P-gp were later harmonized in accordance with a theory proposed by Lee et al. [48], who postulated that P-gp could exist in two dynamic conformations—an open conformation [45] and a closed conformation [47]. Similarly, MRP1 was modeled based on the crystal structure of *Vibrio cholerae* MsbA [46]. The model implied that Phe594 forms a hydrophobic pocket with four residues, therefore successfully

predicting the functional importance of the Phe594 in transmembrane helix 11 of the MRP1 [46].

The transporter-based approach provides detailed protein structural information. Based on the topographical characterization of the protein, one can identify potential areas/pockets where substrate and/or inhibitor interact with the transporter proteins. It should be noted that this approach is not feasible for proteins without a suitable TMD template. To date, this approach has been applied mostly to transporters possessing 7 or 12 TMDs with the exception of MRP1, which has 17 TMDs [37]. For those transporters for which no suitable TMD template is available, techniques such as substrate-based methods that do not require knowledge of transporter structure can be applied.

12.3.2 Substrate-Based Modeling

Substrate-based modeling correlates biological activity of a series of substrates or inhibitors for a transporter with their molecular descriptors or chemical features. Based on the compiled properties of the substrates and inhibitors, the models can then predict potential transporter interactions. This approach has been applied successfully to generate pharmacophore (the spatial arrangement of the hydrophobicity, hydrogen bonding status, charged state, and other features of a compound) and quantitative structure–activity relationship (QSAR) models for many membrane transporter proteins, such as P-gp [49], MRP2 [50], human peptide transporter 1 (hPEPT1) [51, 52], organic cation transporters (OCTs) [53], organic anion transporter polypeptides (OATPs) [54], and nucleoside transporter [55, 56].

Several substrate-based computational programs are commercially available to generate these modelings. The most widely used programs include CATALYST (Accelrys Inc., San Diego, CA) and distance comparison (DISCO) [57] for pharmacophore generation. For more quantitative structure–activity relationship (QSAR) predictions, comparative molecular field analysis (CoMFA) [58] and comparative molecular similarity index analysis (CoMSIA) [59] are commercially available modeling programs. Both CATALYST and DISCO programs attempt to determine common features based on the superposition of active compounds (i.e., transporter substrates or inhibitors) using different algorithms [37]. For example, HIPHOP is one of the algorithms of CATALYST which generates a pharmacophore based on common chemical features among active molecules. In an effort to identify novel hPEPT1 inhibitors, Ekins and co-sworkers [52] used three relatively high affinity ligands—Gly-Sar, bestatin, and enalapril—to generate a common features (HIPHOP) pharmacophore. This consisted of two hydrophobic features, a hydrogen bond donor, acceptor, and a negative ionizable feature. The pharmacophore was then used to search the Comprehensive Medicinal Chemistry (CMC) database of more than 8000 drug-like molecules and retrieved 145 virtual hits mapping to the pharmacophore features. Furthermore, the pharmacophore was also able to identify known hPEPT1 substrates and inhibitors in further database mining of more than 500 commonly prescribed drugs [52]. In contrast to pharmacophore modeling, a 3D-QSAR model consists of a mathematical equation describing potency as a function of 3D interaction fields around aligned training set compounds [60]. Both CoMFA and CoMSIA are commonly used algorithms for 3D-QSAR generation. They are based on the assumption that changes in binding affinities of ligands are related to changes in

molecular properties, represented by interaction fields [58, 59]. Recently, Biegel et al. [61] determined the structural requirements for the substrates of the renal type peptide transporter (PEPT2) by 3D-QSAR using the CoMSIA program. The model was built based on 83 compounds (including dipeptides, tripeptides, and β-lactam antibiotics) covering a wide range of affinity constants. An analysis of the contour maps provided by the CoMSIA method gave insights into the requirements for high affinity substrates of PEPT2. Furthermore, an additional 3D-QSAR model based on the same compounds was generated and correlated with affinity data of the intestinal peptide transporter (PEPT1). By comparing the CoMSIA contour plots, differences in substrate selectivity between PEPT1 and PEPT2 were determined [61].

Substrate-based modeling can often provide insight in the ligand–protein binding or inhibition process and assist in the identification of other molecules that contain the same pharmacophore features. Furthermore, the correlation between the variations in ligand or inhibitor binding affinities and the variations in their respective structural features can assist in the design of more potent inhibitors or substrates with higher affinity. However, disadvantages are also present in the substrate-based modeling approach. First, the training datasets that the models are based on are generated from different sources, that is, different cell type, species, and laboratories. Therefore, the data are often not directly comparable, and the quality and consistency of the data might also be in question. Second, although a large body of literature involving the QSAR of various transporters can be found, it is often based on datasets comprised of transport inhibitors, not necessarily transport substrates. This is especially true for the QSAR modeling of P-gp and MRP, as much of the early work was aimed at finding compounds that could inhibit these transporters and thus reverse multidrug resistance [62]. The major reason inhibition data is frequently used to establish the computational models is because generation of this data is relatively higher throughput and simpler in experimental design compared to determination of the kinetic data (K_m, V_{max}) for transported substrates [37].

12.3.3 Applied Computer Modeling Systems in ADME

Within the ADME process, absorption and elimination (especially through metabolic pathways) have been the most extensively modeled. With respect to absorption, most of the current computational approaches used to predict absorption are based on the assumption that absorption is a passive process. In contrast, computer models for predicting elimination pathways place more emphasis on active processes such as enzyme–drug interactions. Thus, examination of both processes provides a very diverse exposure to the computational models currently employed in early ADME studies.

One of the simplest and most widely used computational approaches to estimate passive absorption or permeation was developed by Lipinski and co-workers in 1997 [63]. The authors selected a compound library consisting of 2287 drug candidates with good absorption properties according to the data of intestinal epithelial permeability and solubility. Based on the physicochemical properties of these compounds, the authors proposed the "rule of five" (ROF), taking into account hydrogen bonding, molecular weight, and lipophilicity.

Although the ROF allows quick screening of many NCEs and provides an initial indication of human intestinal absorption (HIA) of those compounds, there are some weaknesses associated with this model. First, the ROF does not apply to compounds that are subject to active transport processes. For example, therapeutic classes, such as antibiotics, antifungals, vitamins, and cardiac glycosides, consistently fall outside the ROF parameter cutoffs [63] and yet are effectively absorbed in the GI system via various transport systems. Second, the model was created using those compounds with high absorption as its database; only a limited number of compounds with a low absorption were included.

While the ROF is a model more based on the physicochemical properties of a compound, there are several physiological based models that also have great potential for use within both the early and later stages of drug discovery. Two examples of these models are iDEA™ (*in vitro* determination for the estimation of ADME) and GastroPlus™. GastroPlus was developed by Simulations Plus, Inc. (Lancaster, CA; http://www.simulations-plus.com/). It simulates GI absorption and pharmacokinetics for drugs dosed orally or intravenously in humans and animals. The simulation is based on the advanced compartmental absorption and transit (ACAT) model [64], which is an advanced version of the one originally reported by Yu and Amidon [65]. The ACAT model is a flexible physiologically based simulation with nine compartments corresponding to different segments of the digestive tract including stomach, seven small intestinal compartments, and colon. Each compartment is modeled with accurate physiological information regarding volumes, transit times, length, and radius [64]. This model takes into account pH dependency of basic parameters, solubility and permeability, transport of drug material through the GI tract, and absorption of drug through the intestinal wall into the portal vein. In addition to these, GastroPlus provides the ability for customization by allowing absorption constants to be set individually for each intestinal compartment [66]. With this physiologically based simulation software program, the rate, extent, and gastrointestinal location of oral drug absorption can be predicted [67]. For predictions based on measured permeability, the GastroPlus model has been trained to accept values for human jejunal permeability as input. The determination for human permeability (P_{eff}) is based on a combination of experimental data inputs from both *in vivo* human values and *in situ* rat or dog intestinal permeability values. These values then further convert to human values using a built-in correlation [67, 68]. However, because there is no relation between the Caco-2 data and the human P_{eff} with this model, human P_{eff} cannot be predicted using Caco-2 data [67]. Therefore, a preliminary step is transformation based on a correlation when Caco-2 data is used. Normally, a correlation of human P_{eff} against Caco-2 permeability can be built using reference compounds by users before the simulation. By doing this, the Caco-2 permeability is converted to effective human jejunal permeability, P_{eff}, based on the established correlation [68].

Another computational model for the simulation of absorption is iDEA, a predictive ADME simulation system developed by Lion Bioscience Inc. (San Diego, CA; http://www.lionbioscience.com/). This particular model was developed based on a proprietary database that coupled oral bioavailability data from human clinical trials with preclinical *in vitro* data characterizing various parameters of the drug including solubility, Caco-2 permeability, protein binding, and metabolic stability in human cryopreserved hepatocytes. This model can simulate human physiology and

accounts for regional variations in intestinal permeability, solubility, surface area, and fluid movement [66, 68]. iDEA is based on the STELLA (Structural Thinking Experimental Learning Laboratory with Animation) simulation software and comprises structure-based Caco-2 and absorption modules (SBM) and a physiologically based (PBM) absorption module [69, 70]. Therefore, it allows the prediction of Caco-2 permeability or the fraction of dose absorbed (F_a) of drugs from their structures. It also predicts the rate and extent of human oral drug absorption, by taking into account physiological parameters such as intestinal pH, transit time, and blood flow. Since there are large interlaboratory differences in the absolute values of Caco-2 permeability, it may be necessary to "customize" the simulation package to the individual laboratory. This is done by determining the Caco-2 permeability of several marker compounds for comparison the Caco-2 values used in the simulation software package. In doing this, one can determine how Caco-2 permeability values from Lion Bioscience Inc. correlate with the in-house Caco-2 permeability data and modify the simulation model accordingly.

Overall, the computational approaches for predicting oral drug absorption are still in their infancy. Neither the GastroPlus nor the iDEA system has been evaluated completely. Part of the issues with evaluating the extent to which either model predicts drug absorption in the GI tract is the limited access to a large data bank for fraction of dose absorbed. While such information is often available in-house, public access is limited by most pharmaceutical companies due to issues of confidentiality. An inherent limitation with the iDEA system is that compounds suspected to be metabolized or substrates of active transporters or carriers in the intestine have been excluded from model building [67]. To this extent, GastroPlus shows strengths in its ability to integrate additional data, including transport/efflux processes and intestinal metabolism [68]. Recently, Tubic et al. [71] demonstrated that P-gp was involved in the talinolol nonlinear dose-dependent absorption using GastroPlus simulation modeling. Furthermore, it was observed that the results predicted from the model were comparable to the finding of a Phase I dose escalation study [71].

Compared to GastroPlus, iDEA has a simpler operating structure. Once the server is installed, it runs as a web application on the corporate Intranet. Therefore, the deployment is simple and training is not a big issue. In this respect, iDEA could be aimed at use by non-DMPK specialists (e.g., medicinal chemists), who could access the absorption potential of compounds with a quick and easy tool. In contrast to the iDEA system, GastroPlus is well designed and suitable for those scientist dealing on a day-to-day basis with intestinal absorption issues. Because of the more complex modeling parameters, GastroPlus requires more thorough training compared to the iDEA system [68].

12.4 *IN VITRO* SYSTEMS FOR IDENTIFICATION OF TRANSPORTER INTERACTIONS

Identification of transporter interactions with drugs and determination of their impact on the ADME profile at an early stage in drug development has resulted in increased emphasis being placed on relatively high throughput *in vitro* screening methods. Commonly used cell-based approaches for examining transporter

TABLE 12.1 Cell-Based Approaches for Examining Transporter Interactions in the ADME Process

Cell System	ADME Property	Transporter Applications	Experimental Design
Intestinal brush border membrane vesicles	Intestinal absorption	• Identification of substrates/inhibitors • Determination of transporter kinetics	• Uptake studies
Caco-2 cell line	Intestinal absorption	• Identification of substrates/inhibitors • Determination of transporter kinetics • Determination of transporter polarity	• Uptake studies • Permeability studies • Bidirectional permeability studies
Isolated brain microvessel endothelial cells	Blood–brain barrier (brain distribution)	• Identification of substrate/inhibitors • Determination of transporter kinetics	• Uptake studies
Cultured brain microvessel endothelial cells	Blood–brain barrier (brain distribution)	• Identification of substrate/inhibitors • Determination of transporter kinetics • Determination of transporter polarity	• Uptake studies • Permeability studies • Bidirectional permeability studies
Liver canalicular membrane vesicles	Hepatic/biliary excretion	• Identification of substrates/inhibitors • Determination of transporter kinetics	• Uptake studies
Cultured hepatocytes	Hepatic/biliary excretion	• Identification of substrate/inhibitors • Determination of transporter kinetics • Determination of transporter polarity	• Uptake studies • Permeability studies • Bidirectional permeability studies
Madin–Darby canine kidney (MDCK) cell line	General absorption, distribution, and excretion	• Identification of substrate/inhibitors • Determination of transporter kinetics • Determination of transporter polarity	• Uptake studies • Permeability studies • Bidirectional permeability studies

interactions and their applications in ADME processes are listed in Table 12.1. These include isolated cell or membrane preparations from various tissues. Examples are the use of isolated brain microvessel endothelial cells to study transporters at the blood–brain barrier [72, 73] and the use of membrane vesicles prepared from the brush-border membrane of intestinal epithelial cells [74] or the canalicular membrane of liver hepatocytes [75] to study transporter processes in the intestine and liver, respectively. While isolated cell and cell membranes typically have the highest levels of transporter expression, use of these preparations requires a great deal of starting tissue and determination of actual permeability

and polarized transport is difficult. In addition, assay validation with the isolated cell and cell membrane preparations is difficult. Thus, efforts have been made to develop cell culture systems to model the different ADME transport and permeability barriers (see Table 12.1). The advantages of cell culture systems are that one can examine polarized transporter processes and actually evaluate both cellular accumulation and permeability across confluent monolayers. Thus, the influence of transporters on the actual permeability of a compound can be determined. For those cell culture systems that utilize established, immortalized cells lines (i.e., Caco-2, MDCK) there are the added advantages of reproducibility and low interassay variability. In addition, the cell lines can also be transfected to express specific transporters of interest. Examples are the MDCK cell lines that have been transfected with MDR1, MRP1, or MRP2 drug efflux transporters [76–78]. The availability of the transfected cell lines provides important research tools for honing in on transporter dynamics and function.

12.4.1 *In Vitro* Accumulation Assays for Identification of Transporter Processes

Experiments examining the transport properties of compounds in cell-based systems fall into three major categories. These include studies based on cell or vesicle accumulation, unidirectional transport, or bidirectional transport. Studies examining the cellular or membrane vesicle accumulation of a compound can provide a fast and reliable method for identifying both transporter substrates and inhibitors. An example of this is the use of various membrane vesicle preparations to identify the transporter-mediated uptake and efflux of the lipid lowering drug pitavastatin [79]. These studies examined both the acid and lactone forms of pitavastatin. In the case of the acid form, uptake into the cell was dependent on multiple solute transporters, including members of the organic anion transporter (OAT) and the organic anion transporter polypeptide (OATP) family. In contrast, the lactone form was not a substrate for transport into the cell [79]. With regard to transporter-mediated efflux from the cell, the lactone form of pitavastatin was a substrate for P-gp, while the acid form displayed BCRP efflux activity [79]. These studies are a prime example of how vesicular and cellular accumulation can be used to delineate the structure–activity requirements for transport processes.

Cell accumulation assays are also helpful for identification of compounds that can inhibit drug transport. An example is the use of isolated brain capillaries and cultured brain microvessel endothelial cells in drug screening assays for drug efflux transporters [73, 80]. In these studies, changes in the cellular accumulation of fluorescent probes for P-gp and MRP were used to quantitatively assess the inhibitor properties of a series of compounds. Identification of potential inhibitors of drug efflux transport proteins, especially P-gp, are increasingly part of the early testing in the drug development process. This is due to the emphasis placed on knowing whether a compound is a P-gp inhibitor and/or inducer at the time a new drug application (NDA) is filed with the Food and Drug Administration (FDA). In addition, since many inhibitors of drug transport can also act as substrates, cellular accumulation assays are often used as a first screen for potential transporter interactions of compounds. Those agents showing activity can then be examined for substrate properties.

A final application for the cellular accumulation assays is quantitative determination of transporter kinetics. Using the cellular accumulation of fluorescent probes

for P-gp and MRP transport, Bachmeier et al. [81] determined and compared the Michaelis–Menten kinetics of these drug efflux transporters in primary cultured brain microvessel endothelial cells, MDR1 transfected MDCK cells, and freshly isolated brain microvessels. In these studies, P-gp related transport displayed similar K_m values across the different BBB models; however, V_{max} was significantly higher in the freshly isolated brain microvessels [81]. For MRP related transport, differences in both K_m and V_{max} were noted in the various BBB models [81].

12.4.2 *In Vitro* Permeability Assays for Identification of Transporter Processes

Drug interactions with transport systems can also be evaluated using actual cell monolayer permeability. These studies can be performed unidirectionally with and without various transport inhibitors to determine the impact of the transport system on actual permeability of the compound. This type of experiment typically examines the absorptive, distributional, or excretory transport. As with the cellular accumulation assays, transported compounds will display concentration-dependent monolayer permeability. An example is the permeability studies for the amino acid phenylalanine in Caco-2 monolayers [82]. These studies showed that the transport of L-phenylalanine from the apical to basolateral side of Caco-2 cells was concentration dependent. The transport process was saturable with a very high affinity (K_m = 0.019 mM) to the amino acid transporter in Caco-2 cells [82]. Studies performed in the presence of specific transport inhibitors will also influence permeability. For absorptive transporters such as PepT, inhibition of the transporter results in reduced permeability in cell expression of PepT [82]. For drug efflux transporters, such as P-gp, inhibitors will increase the absorptive or distributive permeability of substrates [72, 78]. Alternatively, bidirectional permeability studies can be performed to identify potential transport systems. In these studies permeability is assessed in the apical-to-basolateral (A-B) direction and also in the basolateral-to-apical (B-A). By examining the resulting B-A/A-B ratio of the permeability coefficients, one can determine whether absorptive/uptake transporters (ratios significantly less than 1) or efflux transporters (ratios significantly greater than 1) are involved. Bidirectional permeability studies in either Caco-2 or MDCK MDR1 cells have been very useful for identifying those compounds that are transported by drug efflux transport proteins [83, 84]. While most commonly used to identify secretory transport systems, bidirectional permeability studies are also useful for the identification of absorptive transport properties as well. Studies by Li and colleagues [85] used bidirectional permeability in Caco-2 cell monolayers to aid in the identification of amino acid prodrugs of the antiviral levovirin, which utilize the PepT system. These studies were able to identify ester-based amino acid prodrugs with PepT substrate and inhibitor properties. Such compounds are anticipated to result in improved oral bioavailability for levovirin.

12.5 *IN VIVO* METHODS FOR ASSESSING TRANSPORT INTERACTIONS

Examination of drug transporter interactions at the whole animal level is much more complex than cell-based assays. However complex, ultimate determination of

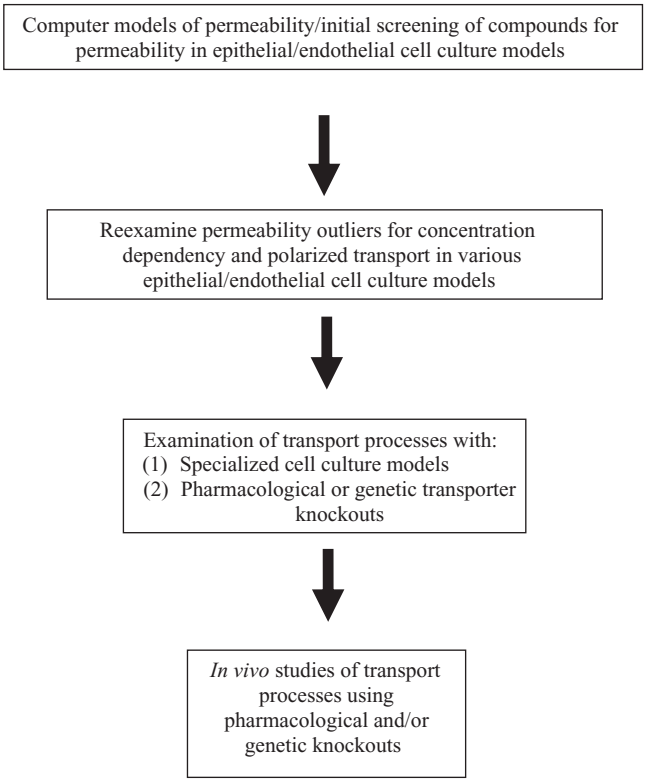


FIGURE 12.1 Experimental process for determining transporter-related processes in ADME.

the impact that transporters have on the ADME properties of a drug require whole animal studies. Thus, *in vivo* studies examining transporter influences on the ADME properties of a drug are typically performed after extensive *in vitro* evaluation of the transport properties of the lead compound(s) (see Fig. 12.1). The exception to this is the cassette dosing of drugs to rodents during the early drug development stage. In these studies, a series of compounds are administered together, to obtain an early glimpse of the ADME properties in the *in vivo* setting [86–88]. However, it should be noted that performing the cassette dosing at both low and high doses of the compounds can provide preliminary indications of drug transporter interactions, as drugs that are substrates for transporters will demonstrate concentration dependency [88].

The advantage to performing *in vivo* studies during the final stages of the preclinical development of a drug is that a great deal of information concerning drug transporter interactions has already been identified. This allows the *in vivo* studies to hone in on the appropriate transporter using either pharmacological or genetic inhibition of transport properties. In the past, *in vivo* demonstration of transport relied on dose dependency and the use of pharmacological agents that inhibited transport processes. However, through advances in molecular biology, researchers now have the ability to selectively "knock out" drug transporters either through

creation of transgenic animals [89] or, more recently, through the use of small inhibitory RNA (siRNA) [90], to transiently knock down transporter expression. An example of the power that transgenic knockouts bring to *in vivo* studies of transporter interactions in the ADME process can be found in the MDR1 transgenic knockout mice [89, 91]. Studies with these mice have established quite conclusively the influence that P-glycoprotein has in the oral absorption, brain penetration, and elimination of selected drugs [91]. Similar examples can be found with the PEPT2 knockout mice, where levels of model PEPT2 substrates have been reduced in blood and cerebral spinal fluid owing to the lack of transporter at the kidney and blood–cerebrospinal fluid barriers [92].

The use of transgenic animals for studying ADME processes is not without its limitations. This is especially true when dealing with transporters. The main problems with transgenic animals in the study of ADME properties of a drug revolve around the related issues of transporter promiscuity and transporter compensation. For many transporters, there are various subtypes or homologs. Examples include the MRP drug efflux transporter family with over nine different homologs [93–95], and the organic anion transporter (OAT) with at least five homologs in humans [96]. Given the overlapping substrate and inhibitor properties of these transporters [17, 95] it is no wonder that transgenic knockout mice for single homologs of *mrp* have not yielded the dramatic results observed with *mdr1* knockout mice [97, 98]. The other issue to keep in mind is the upregulation of compensatory transport systems in the various transgenic knockout models. For example, *mdr1* knockout mice deficient in P-gp appear to have enhanced BCRP transport. This is suggested by the studies of Salama and colleagues [99], which evaluated the tissue accumulation of the HIV protease inhibitor nelfinavir, in wild-type and *mdr1* knockout mice. While significant increases in the brain accumulation of nelfinavir were observed in the *mdr1* knockout mice compared to wt controls, an even greater increase was observed when the P-gp/BCRP inhibitor GF120918 was administered to the knockout mice. As these mice had no P-gp, the increased brain accumulation of nelfinavir in the *mdr1* knockout mice exposed to GF120918 is likely due to inhibition of BCRP. These findings are consistent with the studies of Cisternino and colleagues [100] that show an increased expression of BCRP in brain microvessel endothelial cells from *mdr1* knockout mice compared to wt mice, and suggest compensatory increases in BCRP can occur in selected tissues as a result of P-gp deficiency.

In vivo application of siRNA to selectively knockdown transporter expression is relatively new. The technique involves the introduction of single chain oligonucleotides into the cell that bind to specific single chain RNA sequences preventing transcription into protein [90]. The use of siRNA to knock down the expression of a variety of protein targets has been demonstrated in cell culture systems [90]. While there are reports in the literature of successful use of siRNA technology *in vivo*, applications to transporter relevant topics are lacking. The main obstacle to application of siRNA technology for the study of ADME-related transport processes is the effective delivery of siRNA to the relevant tissue/organs [101]. However, as methods for the efficient introduction of siRNA into targeted tissue improve, it is highly likely that this technique will have future applications for ADME-related studies in the whole animal.

12.6 CONCLUSION

The increasing cost of drug development is partially due to the failure to identify undesirable compounds at an early enough stage of the drug discovery process. Therefore, development of tools capable of predicting the ADME (absorption, distribution, metabolism, and excretion) properties of the new chemical entities (NCEs) becomes a significant way to reduce the cost for drug failure and to improve the chances of designing drugs with optimal ADME characteristics. In this regard, *in vitro* models capable of screening large numbers of compounds and computational (*in silico*) based models have greatly streamlined the early ADME process. This is especially true for identification of transporter interactions at the early stages of drug development. Using a rationale approach, such as that outlined in Fig. 12.1, that incorporates early *in silico* and cell-based assays to identify potential compounds that interact with transport systems coupled with *in vivo* studies of the ADME properties of identified compounds, one can optimize the identification of transporter-related issues and evaluate their potential impact on the ADME properties of drugs in the developmental pipeline.

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