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In Vivo Approaches to Assessing the Blood–Brain Barrier

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Abstract Methods for in vivo assessment of blood-brain barrier (BBB) transport are presented, with their advantages and disadvantages. The methods described are brain uptake index, the i.v. injection technique, in situ brain perfusion, brain efflux index, % injected dose, microdialysis, CSF sampling and positron emission tomography, and the combinatorial mapping of unbound drug partitioning across the BBB. The methods are put into a pharmacokinetic context by delineating the type of readings that they give, be it the rate of transport across the BBB or the extent of transport of total drug (unbound and bound), or of the unbound drug.

Keywords Brain uptake index, i.v. injection technique, In situ brain perfusion, Brain efflux index, Microdialysis, CSF sampling, Positron emission tomography, Fraction unbound in the brain, Brain homogenate method, Brain slice technique, Volume of distribution of unbound drug in the brain

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Abbreviations

%ID	Percentage of the injected dose
A	Capillary surface area (also denoted S in the literature)
AUC	Area under the concentration–time curve
BBB	Blood–brain barrier
BCRP	Breast cancer resistance protein
BCSFB	Blood–cerebrospinal fluid barrier
BEI	Brain efflux index
BUI	Brain uptake index
C_{blood}	Concentration of drug in blood
C_{brain}	Concentration of drug in brain devoid of blood
$C_{injectate}$	Concentration of drug in the injection solution
C_{plasma}	Concentration of drug in plasma
CL_{act_efflux}	Active efflux clearance at the BBB (sum of all processes contributing to active efflux)
CL_{act_uptake}	Active uptake clearance at the BBB (sum of all processes contributing to active uptake)
CL_{bulk_flow}	Clearance caused by bulk flow of fluid from brain ISF to CSF
CL_{in}	Influx clearance i.e. the net influx given all transport processes at the BBB
$CL_{metabolism}$	Clearance caused by metabolism in the BBB or brain parenchyma
CL_{out}	Efflux clearance, i.e., the net efflux given all transport and metabolism processes from the brain ISF
$CL_{passive}$	Passive clearance (permeability surface area product) across the BBB being the same in both directions
CSF	Cerebrospinal fluid
$C_{tot,brain,ss}$	Total brain concentrations at steady state (whole brain minus capillary blood)
$C_{tot,plasma,ss}$	Total plasma concentrations at steady state
$C_{u,brainISF}$	Concentration of drug in brain ISF
$C_{u,plasma}$	Unbound drug concentration in plasma
F	Blood flow
$f_{u,brain}$	Fraction of unbound drug in whole brain homogenate
$f_{u,plasma}$	Fraction of unbound drug in plasma
ICF	Intracellular fluid
ISF	Interstitial fluid
J_{in}	Rate of influx to the brain
J_{out}	Rate of efflux from the brain
K_{in}	Transfer constant at the BBB (a clearance term)

K_{out}	Overall loss constant (a clearance term)
$K_{p,brain}$	Partition coefficient of total drug between whole brain and plasma
$K_{p,uu,brain}$	Partition coefficient of unbound drug between brain ISF and plasma
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
Mrp1	Multidrug resistance protein 1
P	Permeability
PET	Positron emission tomography
P-gp	P-glycoprotein
PA	Permeability surface area product (mL min^{-1} g $brain^{-1}$) also denoted PS
Q_{brain}	Amount of drug in brain parenchyma devoid of blood
$Q_{tot,brain}$	Amount of drug in brain parenchyma including capillary blood
V_{blood}	Physiological volume of blood in brain
V_{brain}	Effective volume of distribution in the brain
V_i	Effective vascular space in which a compound can be found including endothelial cell binding and accumulation and intravascular volume
$V_{u,brain}$	Volume of distribution of unbound drug in the brain

1 Introduction

The blood–brain barrier (BBB) is an intricate organ that is made up of the endothelial cell walls of the brain capillaries, thus extending throughout the whole brain. The length of the capillary network in one human brain is 644 km, the surface area is 20 m^2, and the distance between two capillaries is no more than 25–40 μm, while the thickness of the wall is one cell layer or 200–500 nm [1]. The function of the BBB is to control the environment of the brain by promoting the uptake of nutrients, hindering the entrance of harmful compounds, and effluxing metabolites. Much has been discovered regarding the functions of the BBB in recent years. The focus is currently on the whole neurovascular unit, which consists not only of the endothelial cells, but also encompasses the astrocytes, pericytes, basement membranes, and surrounding connections to neurons and glial cells. It is becoming clearer that all these components collaborate to maintain a tight, well-functioning system of exchange with the blood compartment [2–4].

There are several approaches to the assessment of BBB function; many involve in vitro cell culture models which allow the different mechanisms of BBB function to be studied in detail. These approaches will not be discussed in this chapter, but some references are provided for further reading [5–22].

In vivo approaches to studying the BBB involve estimation of drug concentrations in both brain and blood and include such procedures as microdialysis and the i.v. injection technique, in situ brain perfusion, and the brain efflux index (BEI) method. The concentration of unbound drug in the cerebrospinal fluid (CSF) may also be used as a replacement for that in the brain. Several review articles and book chapters have discussed in vivo methods of studying the BBB [11, 12, 23–29].

Because the reasons for studying the BBB differ, different methods are required. Either endogenous or exogenous substances can be studied. In this chapter, the focus is on the study of the rate and/or extent of transport of exogenous compounds such as drugs across the BBB. In many cases, these same methods can be used for studying endogenous compounds.

This chapter will therefore outline the available in vivo and in situ methods of assessing BBB function, listing some of their specific properties. The pharmacokinetic principles behind the BBB transport of drugs are presented first, followed by discussion of the various methods and the properties of BBB transport that each illustrates.

2 Pharmacokinetic Principles of Blood–Brain Barrier Transport

The driving force for drug transport to the brain is the concentration of unbound drug in the plasma ($C_{u,plasma}$) (Fig. 1). After administration, the drug molecules in the plasma will endeavor to achieve equilibrium with all body tissues, including the brain. Molecules will be let through the BBB from the plasma to the brain passively, will be effluxed if they are the substrates of efflux transporters like P-glycoprotein (P-gp) or breast cancer resistance protein (BCRP), or will be actively taken up into the brain by influx transporters at the BBB [30, 31]. Once in the brain interstitial fluid (ISF), the drug molecules are distributed into the intracellular fluid (ICF) and may bind specifically or nonspecifically to components of the brain parenchymal cells. The drug molecules that are present in the brain ISF are defined as being unbound. The brain ISF accounts for 19% of the total volume of brain tissue [32]. Most drug binding takes place in the intracellular compartment, as intracellular membranes provide a majority of all membranes in the brain parenchyma.

The free drug hypothesis states that only unbound drug molecules can interact with receptors. Thus, the drug molecules that are bound to plasma proteins or to components of the brain parenchyma are not pharmacologically active but act as a pool to and from which drug molecules are bound and released. Experimental evidence indicates that the brain unbound drug concentrations predict receptor binding or pharmacological effects much more reliably than the total brain or even unbound drug plasma concentrations; this was most clearly demonstrated by Watson and coworkers for dopamine D_2 receptor occupancy of antipsychotic drugs [33]. When a drug target is situated within the membranes, it is more difficult to predict whether the unbound or bound drug molecule is the active entity but, irrespective of the direct action, it is the unbound drug that equilibrates across the different compartments (Fig. 1).

Drugs can have very different affinities to brain parenchymal tissue. Thus, the total concentration of drug measured in the whole brain can differ substantially from the concentration of unbound, pharmacologically active drug. Total brain

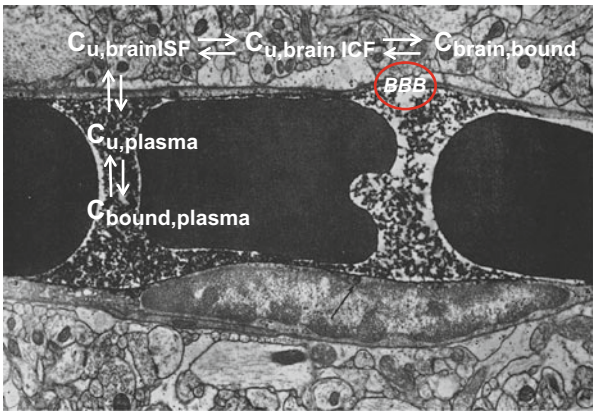

Fig. 1 Distribution of drugs in plasma and brain across the endothelial cells comprising the BBB. Further distribution takes place into the brain interstitial fluid (ISF) and brain intracellular fluid (ICF). The equilibria between the different sites are projected on an electron micrograph of a brain capillary depicting three red blood cells (*black*), endothelial cells (marked with a *red circle* at the top right and BBB), a pericyte, and brain parenchymal cells surrounding the capillary [90]. With permission from Rockefeller University Press. © 1967 Reese and Karnovsky. Originally published in The Journal of Cell Biology 34:207–217

concentrations can be 1- to 3,000-fold higher than unbound drug ISF concentrations [34]. Total brain concentrations could therefore be up to 3,000-fold higher than the actual concentration of active moiety required for therapeutic success.

The unbound drug concentrations in plasma can differ substantially from the unbound drug concentrations in the brain, and plasma concentrations are therefore not suitable for predicting the effects. The differences between plasma and brain unbound drug concentrations are the result of the active transporters in the BBB, which dramatically and substantially influence the concentrations in the brain but are not yet predictable using in vitro methods. The movement of drugs across the BBB can be influenced either by efflux or influx transporters or by a combination of transporters acting on one drug. The influence of these transporters can result in brain unbound drug concentrations ranging from less than 1% of the corresponding plasma concentrations up to five times these concentrations [30, 31, 35]. The term used to depict the steady-state ratio of unbound drug in brain ISF to that in plasma is the partition coefficient $K_{p,uu,brain}$ [36, 37].

$K_{p,uu,brain}$ describes the *extent of transport*, or rather the extent of equilibration, across the BBB. It is determined by the balance of transport into and out of the brain tissue. Both processes mainly take place through the BBB, although metabolism within the brain parenchyma and bulk flow of fluid from the ISF to the CSF can also contribute. Bulk flow into the CSF could contribute more significantly to the efflux of drugs which permeate poorly across the BBB [38, 39].

The *rate of transport* of a drug into or out of the brain is described in terms of the permeability of the BBB to the drug in question. The parameter usually used to describe the rate of transport in vivo is the permeability surface area product

(abbreviated to PA or PS; mL min^{-1} g $brain^{-1}$). The influx PA rates at the BBB can span a large range; for example, for opioids, the influx PA ranges from very low at 1.1×10^{-4} mL min^{-1} g $brain^{-1}$ for morphine-3-glucuronide to relatively high at 1.9 mL min^{-1} g $brain^{-1}$ for oxycodone [30, 40–42].

In pharmacokinetic terms, the flow into and out of the brain can be expressed in terms of the influx clearance (CL_{in}) and the efflux clearance (CL_{out}) [37]. These are the *net* clearances in each direction, i.e., the sum of all processes at the BBB or in the brain for the drug in each direction. The influx clearance has also been expressed as the transfer coefficient K_{in} and the overall loss from the brain as K_{out} [43–46]. CL_{in} is thus the same as K_{in}, and CL_{out} is the same as K_{out}. The overall influx and efflux rates for a drug, often expressed as J_{in} and J_{out}, include the concentration of drug in the plasma or ISF and can then be described as

$$J_{in} = K_{in} \times C_{u,plasma} \tag{1}$$

$$J_{out} = K_{out} \times C_{u,brain} \tag{2}$$

where $C_{u,plasma}$ and $C_{u,brain}$ are defined as the unbound-compound concentrations in plasma and in brain ISF, respectively [47]. Further equilibration within the brain from brain ISF to the intracellular compartment and from unbound to bound compound takes place according to Fig. 1. Michaelis–Menten kinetics are used if there is a saturable transport process involved. The rate of change in the amount of a compound in the brain under linear conditions is described by

$$\frac{dQ_{brain}}{dt} = J_{in} - J_{out} = \left(K_{in} \times C_{u,plasma}\right) - \left(K_{out} \times C_{u,brain}\right) \tag{3}$$

where Q_{brain} is the amount of compound present in the brain parenchyma apart from in the brain capillaries. $K_{out} \times C_{u,brain}$ in Eq. (3) can be expressed as $k_{out} \times Q_{brain}$, where k_{out} is the rate constant equal to K_{out}/V_{brain}, and V_{brain} is the effective volume of distribution of the compound in the brain (expressed in mL g $brain^{-1}$) [47], which in turn is equal to the volume of distribution of unbound compound in the brain, $V_{u,brain}$ (see below):

$$\frac{dQ_{brain}}{dt} = J_{in} - J_{out} = \left(K_{in} \times C_{u,plasma}\right) - \left(k_{out} \times Q_{brain}\right) \tag{4}$$

At very early time-points, the influence of this term in Eq. (4) is very small, as there is as yet very little drug in the brain. This fact has been used to look at initial uptake [43, 45]. Equation (4) can then be simplified to

$$\frac{dQ_{brain}}{dt} \approx K_{in} \times C_{u,plasma} \tag{5}$$

By further integration of the equation [44, 47, 48], it is possible to determine K_{in} as

$$K_{in} \approx Q_{brain} / \int C_{u,plasma} dt \tag{6}$$

Although Q_{brain} refers to the amount of drug in the brain minus the brain capillary contents, the measurements are often made on the whole brain concentration including the blood ($Q_{tot,brain}$). Compensation for the amount of solute present in the blood is therefore needed. Further development of the equation (see [47]) results in the Patlak equation [44]

$$Q_{tot,brain} / C_{u,plasma} \approx K_{in} \left[\int C_{u,plasma} dt / C_{u,plasma} \right] + V_i \tag{7}$$

where V_i is the effective vascular space in which the studied compound could be found, including endothelial binding. Another, more practical way of expressing the Patlak equation is

$$K_{in} \approx \left(Q_{tot,brain} - V_{blood} \times C_{blood} \right) / \int C_{u,plasma} dt \tag{8}$$

where V_{blood} is the volume of blood in the brain, often measured using an impermeable vascular marker such as [^{14}C]dextran or [^{3}H]inulin, and C_{blood} is the total concentration of the compound in the blood.

Three parameters influence the clearance of drugs from the capillaries: the rate of blood or plasma flow (F), the capillary surface area (A), and the permeability of the capillaries to the solute (P). Thus, K_{in} is not a permeability coefficient, but an in vivo clearance parameter. The relationship was derived by Renkin [49] and Crone [50] as

$$K_{in} = F \left[1 - \exp^{-PA/F} \right] \tag{9}$$

This equation is called the Crone–Renkin equation. Smith has evaluated the limiting conditions for K_{in} [46]. When F is much larger than PA, K_{in} approaches PA in value, and when F is much smaller than PA, K_{in} approaches F in value. This means that the upper limit of K_{in} is the rate of capillary blood flow and the lower limit is the permeability surface area product. It has been suggested that K_{in} can be used to estimate PA when PA is lower than F by a factor of at least 5. PA can then be estimated by rearranging the Crone–Renkin equation (Eq. (9)) as

$$PA = -F \ln(1 - K_{in}/F) \tag{10}$$

F can be estimated using radioactive iodoantipyrine, microspheres, or diazepam [47].

At equilibrium, the rate of solute transport in each direction across the BBB is similar, i.e., $J_{in} = J_{out}$. Using Eq. (3) with clearance terminology, this gives [37]

$$CL_{in} \times C_{u,plasma} = CL_{out} \times C_{u,brainISF} \tag{11}$$

and, thus, the extent of transport can be described as

$$\frac{C_{u,brainISF}}{C_{u,plasma}} = \frac{CL_{in}}{CL_{out}} = K_{p,uu,brain} \tag{12}$$

Equation (13) describes the intricate collaboration between the different transport processes that results in $K_{p,uu,brain}$, showing that $K_{p,uu,brain}$ describes the balance between all influx and efflux processes:

$$\begin{aligned} K_{p,uu,brain} &= \frac{CL_{in}}{CL_{out}} \\ &= \frac{CL_{passive} + CL_{act\_uptake} - CL_{act\_efflux}}{CL_{passive} - CL_{act\_uptake} + CL_{act\_efflux} + CL_{bulk\_flow} + CL_{metabolism}} \end{aligned} \tag{13}$$

In this equation, $CL_{passive}$ describes the passive movement of the drug across the BBB, assumed to be the same in both directions; CL_{act_uptake} describes the sum of the active uptake transport processes; CL_{act_efflux} describes the sum of the active efflux transport processes; CL_{bulk_flow} describes the bulk flow; and $CL_{metabolism}$ describes the removal of the drug from brain tissue or the BBB by metabolism.

For a drug transported across the BBB mainly by passive transport, $K_{p,uu,brain}$ equals unity (Fig. 2). If the active efflux of a drug is faster than the influx, $K_{p,uu,brain}$ will be lower than unity. The lower the value, the more influential is the active efflux process. If $K_{p,uu,brain}$ is higher than unity, there is a net influx of the drug. To date, $K_{p,uu,brain}$ has been estimated to range from <0.01 for drugs like loperamide, methotrexate, and paclitaxel to 5 for diphenhydramine [31, 34].

The extent of delivery of drugs to the brain can also be measured as the total brain concentration at steady state ($C_{tot,brain,ss}$) divided by the total plasma concentration at steady state ($C_{tot,plasma,ss}$), i.e., $K_{p,brain}$ (also known as logBB). This ratio includes any binding of the drug that occurs in the brain and/or plasma and can be expressed in relation to $K_{p,uu,brain}$ as

$$K_{p,brain} = \frac{C_{tot,brain,ss}}{C_{tot,plasma,ss}} = \frac{C_{u,brainISF}/f_{u,brain}}{C_{u,plasma}/f_{u,plasma}} = K_{p,uu,brain} \times \frac{f_{u,plasma}}{f_{u,brain}} \tag{14}$$

$K_{p,brain}$ is thus influenced by three independent properties [37]: the intra-brain binding, as described here by the fraction of unbound drug in the brain ($f_{u,brain}$), the fraction of unbound drug in plasma ($f_{u,plasma}$), and the BBB transport (described by $K_{p,uu,brain}$). The $f_{u,brain}$ parameter needs to be compensated for pH partitioning into acidic organelles, mainly lysosomes [51]. Alternatively, and preferably, the unbound drug volume of distribution in the brain, which can be expressed as

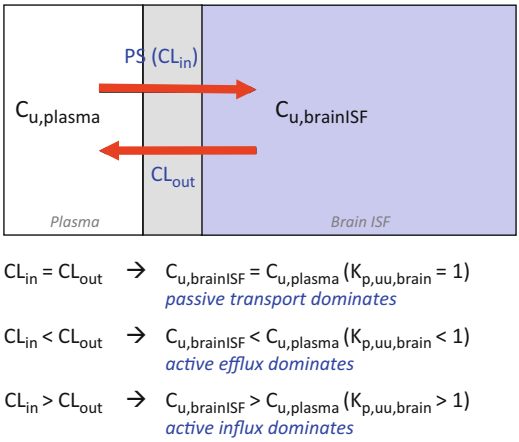

Fig. 2 Interplay between influx and efflux processes at the BBB leading to different unbound drug concentrations in the brain ISF ($C_{u,brainISF}$) from the unbound drug concentrations in plasma ($C_{u,plasma}$). This ratio is the $K_{p,uu,brain}$ (see also Eq. (12)). $K_{p,uu,brain}$ is thus not determined by the absolute values of the influx or efflux clearance, but by the relationship between the two

$1/V_{u,brain}$ in Eq. (14), should be used (see Sect. 3.9). $K_{p,brain}$ is therefore a composite parameter that is not optimal for determining whether a new drug is able to reach the brain in sufficient quantities. The higher the binding in the brain vs that in plasma, the higher the $K_{p,brain}$ value. At the same time, the more efficient the efflux, the lower the $K_{p,brain}$.

3 Methods

The methods described below and in Table 1 are used to estimate either the rate of transport of the drug across the BBB, by measuring PA, or the extent of transport. Some methods are able to measure both properties. The methods that measure the rate of initial unidirectional uptake of drug at the BBB are generally not influenced by elimination from the brain. They are, however, influenced by active processes at the BBB (both influx and efflux) in addition to passive transport. They therefore measure the rate of net uptake or net efflux.

The methods for measuring BBB transport have been described by several authors [11, 23, 27, 47, 52]. The review by Smith et al. is very insightful and offers much additional information on the methods presented here [47]. Additional methodological issues associated with some of these methods have also been discussed by Hammarlund-Udenaes [53].

Table 1 Overview of methods used for studying the rate and/or extent of BBB drug transport

Method	Property
Brain uptake index	In principle a rate method, but does not measure PA
i.v. injection technique	Rate method (K_{in})
In situ brain perfusion	Rate method ($K_{in} \rightarrow$ PA)
Brain efflux index	Rate method ($k_{el} \rightarrow CL_{efflux}$)
Percentage of injected dose	Extent method
Microdialysis	Extent method ($K_{p,uu}$); rate method if data are modeled and $V_{u,brain}$ is measured
Brain-to-plasma ratio of total drug concentrations	Extent method (K_p); a composite parameter using total drug concentrations
Brain-to-plasma ratio of unbound drug concentrations	Extent method ($K_{p,uu}$); maps BBB transport using unbound drug concentrations
CSF sampling	Extent method; but estimates CSF-to-blood partitioning and not necessarily brain-to-blood transport
Positron emission tomography	Measures both the rate and extent of transport; uses total drug concentrations
Brain slice method measuring $V_{u,brain}$	Neither rate nor extent of transport; measures intra-brain distribution
Brain homogenate method measuring $f_{u,brain}$	Neither rate nor extent; measures intra-brain distribution

3.1 *Brain Uptake Index (Carotid Artery Single Injection Technique)*

The brain uptake index (BUI) provides an estimate of the rate of uptake of drug injected into the brain in relation to the rate of uptake of a reference compound. In the original publication, the BUI technique was called the carotid artery single injection technique [54]. A radioactively labeled reference compound that is freely diffusible across the BBB, often ^{3}H-water, ^{3}H-diazepam, or ^{14}C-butanol, is rapidly (0.5 s) injected into the common carotid artery in about 0.2 mL of buffered Ringer's solution. The animal is decapitated 5–15 s after administration. The assumptions are that there is no transport of drug from brain to blood and that there is no metabolism during the time of the experiment. The BUI is calculated according to Eq. (15) as

$$BUI = \frac{\left(C_{brain}/C_{injectate}\right)_{test}}{\left(C_{brain}/C_{injectate}\right)_{reference}} \times 100 \quad (15)$$

where C_{brain} is the concentration of the drug in the brain devoid of blood, and $C_{injectate}$ is the concentration of the drug in the injected buffer. Figure 3 shows the BUI for four opioids, demonstrating one of the disadvantages associated with this technique (see also Table 2). As the transit time through the brain capillaries is very short (1 s), there is too little time for morphine to be transported into the brain. Thus,

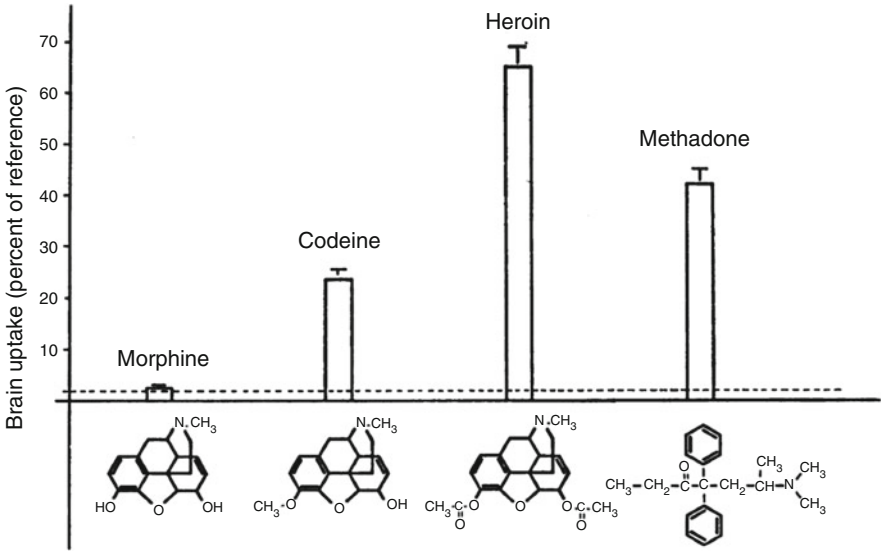

Fig. 3 Classical figure of the brain uptake index of four opioids. A radioactively labeled drug is injected into the carotid artery with ^{3}H-water or ^{14}C-isopropanol as the diffusible reference compound and a sample is taken at 15 s. From Oldendorf et al. [91] with permission from the publisher

Table 2 Advantages and disadvantages of the BUI method for studying BBB drug transport

Advantages	Disadvantages
Technically easy	Only provides relative uptake compared to a reference compound
Rapid (sampling after 5–15 s)	Only 10% of compound reaches the brain, which decreases the detection limit
	Short capillary transit time (1 s) precludes PA $<$ 10 μL min^{-1} g^{-1} being measured

morphine is on the limit of detection while heroin has the highest BUI value. In general, PA values below 10 μL min^{-1} are difficult to measure with this method [52]. As a technical caveat, the administered compounds may be transported to the rest of the body and only 10% of the compound might reach the brain [23]. This lowers the detection limit of the method. The BUI method is now considered less useful than, for example, the i.v. injection technique or the in situ brain perfusion technique presented below.

3.2 The i.v. Injection Technique

The aim of the i.v. injection technique is to measure the rate of unidirectional uptake of a molecule into the brain (K_{in}). The method was first published by Ohno

Table 3 Advantages and disadvantages of the i.v. injection technique for studying BBB drug transport

Advantages	Disadvantages
Low technical difficulty (no access to carotid artery needed)	Compensation for metabolite concentrations in blood (and brain) is needed because of the longer times for sampling (easy if LC-MS/MS is used, but difficult if radioactivity is used)
Relatively sensitive; can measure poorly permeating compounds with PA < 0.5 μL min^{-1} g^{-1}	The assumption that only unidirectional uptake is taking place is probably violated because of the relatively long sampling time
Independent of cerebral blood flow (F) when PA $\ll F$	
Both plasma and brain pharmacokinetics can be obtained	
Studies an intact system	

and coworkers [43]. It is currently considered the gold standard for BBB transport studies [47]. Patlak et al. developed the method to allow graphical representation and multiple time-point measurements [44]. They also discussed aspects of the methodology that required optimizing [48].

Equations (4)–(10) describe this method, which can be used to estimate K_{in}, k_{out}, V_{brain}, F, and PA. There are no assumptions made regarding the intra-brain distribution, but it is assumed that no elimination from the brain takes place during the measurement.

Briefly, an i.v. bolus containing the drug is injected into the femoral or tail vein of the model animal. Samples are then taken serially from the femoral artery. The brain and sometimes also the CSF are sampled at the last time-point. Alternatively, only one arterial blood sample is taken at the same time as the brain sample. It is possible to study the process over a period of less than a minute to hours; however, most studies are no longer than 60 min. This extended time-span violates the assumption of unidirectional uptake, as the compound studied is able to be transported back from the brain to the blood in possibly quantitatively important amounts. Compensation for metabolite formation is also necessary.

The use of the i.v. injection technique to measure the PA is probably not as relevant today as it has been; the available improved cell models can more easily measure the PA without the need for animals. However, cell models are not fully able to describe the in vivo situation to estimate the other parameters, and this is where the i.v. injection technique is of value (Table 3).

3.3 In Situ Brain Perfusion

The in situ brain perfusion method also measures the rate of transport, providing PA and F via measurement of K_{in} (see Eqs. (5–10)). It was developed by Takasato

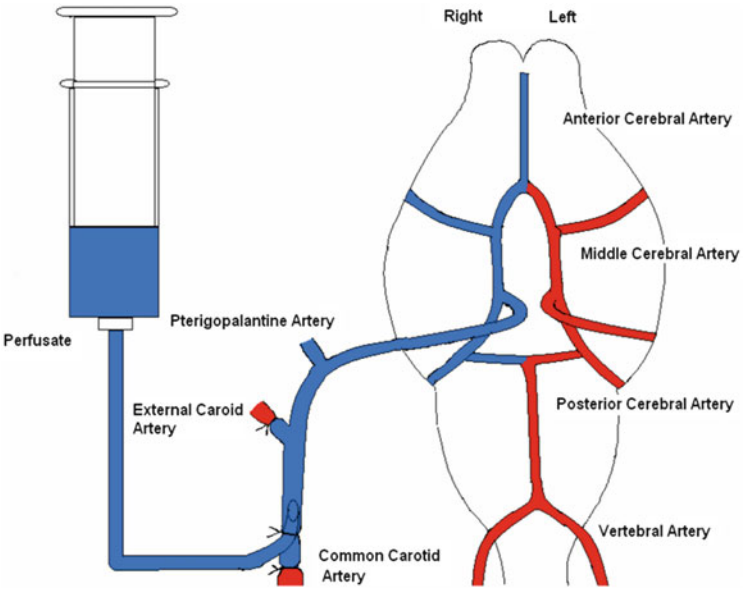

Fig. 4 Surgical procedure and perfusion in the in situ brain perfusion method. From the thesis by H. Mandula, Texas Tech University, 2005, with permission from the author

et al. [45] and was described in detail by Smith and Allen [55]. The method has been further developed by others for studies in mice [56–58].

The procedure is performed in anesthetized rats or mice. The ipsilateral pterygopalatine, superior thyroid, and occipital arteries are ligated and cut. The external carotid artery is ligated and the perfusion catheter is placed either in the external carotid artery [45] or directly in the common carotid artery distal to the bifurcation of the common carotid artery, as in Fig. 4. The ipsilateral common carotid artery is then ligated. The perfusion fluid flows towards the brain at a rate of 3.5–4 mL min^{-1} (some sources say 5–20 mL min^{-1}). This is to produce an arterial pressure equal to the systolic pressure to prevent the perfusate mixing with the circulating rat plasma within the cerebral circulation. D-glucose is added to provide energy. The perfusion can be sustained from 5 s to 10 min [55], but is normally no longer than 120 s.

A reference compound is perfused with the compound(s) of interest, to measure the brain plasma volume. Radiolabeled sucrose or inulin is often used for this purpose [55]. After perfusing the compound of interest and the reference compound, a physiological buffer can be perfused for 10–30 s to separate the bound and transcytosed compounds [55].

The in situ brain perfusion method is more sensitive than the BUI method because the experimental time is longer and the vessels that do not lead to the brain are ligated, thereby resulting in 100% of the perfused solution entering the brain (Table 4).

Table 4 Advantages and disadvantages of the in situ brain perfusion technique for studying BBB drug transport

Advantages	Disadvantages
Rapid	Technically challenging
More sensitive than BUI	Unsuitable for high-throughput use
Lack of systemic exposure of the compounds studied means no influence from peripheral metabolism	
The composition and flow rate of the perfusate fluid can be fully controlled	
Provides mechanistic information	
Competitive processes at the BBB can be studied	
Negligible mixing of perfusion fluid with blood	

Table 5 Advantages and disadvantages of the brain efflux index method for studying BBB drug transport

Advantages	Disadvantages
Can measure carrier-mediated transport of both small and large molecules	Technically challenging
	Very small injection volumes

3.4 Brain Efflux Index

The BEI method is used to characterize the rate of efflux transport from the brain (cerebrum) to the blood across the BBB, and to describe the relative amounts of test and reference compounds effluxed (using k_{el} to estimate CL_{efflux}). The method was developed by Kakee et al. [59, 60]. This and other methods were used by Ohtsuki et al. to study the elimination of the uremic toxin indoxyl sulfate and various neurotransmitters from the brain to the blood via the transporter OAT3 [61]. The advantages and disadvantages of the method are presented in Table 5.

Briefly, anesthetized rats are placed in a stereotactic frame. The skull is exposed and a hole is burred so as to place the cannula in the PAR2 (cortex) region (Fig. 5). A radiolabeled compound is microinjected together with a nonpermeating reference compound (which will remain in the brain parenchyma) in a volume of 0.1–1 μL. It is very important that the injection and retraction of the needle are performed very slowly. The PAR2 region was chosen because it allows minimal diffusion into the rest of the brain. [^{14}C]carboxyinulin can be used as the reference compound for [^{3}H]-labeled compounds, and [^{3}H]inulin, [^{3}H]dextran, or [^{3}H]D-mannitol can be used for [^{14}C]-labeled compounds. Brain and plasma are sampled at various times after the injection to provide an elimination profile (Fig. 6).

The BEI is defined as the relative percentage of drug injected into the cerebrum that is effluxed from the ipsilateral cerebrum to the blood:

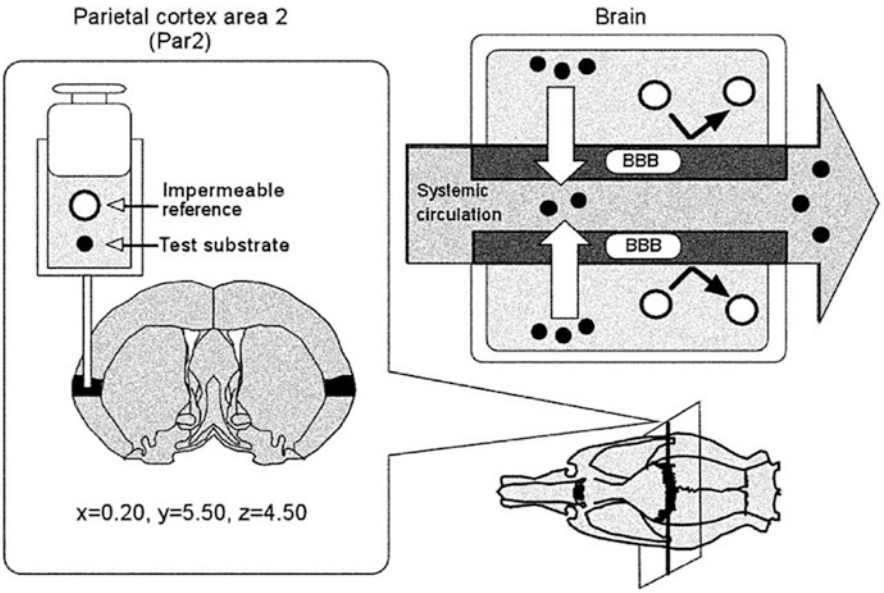

Fig. 5 Depiction of the brain efflux index method showing placement of the co-injection of an impermeable reference compound and the test substrate in the parietal cortex area 2 of the rat. The *top right* picture shows the elimination of the test compound while the reference stays within the tissue. From Hosoya et al. [92] with permission from the publisher

$$\text{BEI } \% = \frac{\text{Compound effluxed at the BBB}}{\text{Compound injected into the brain}} \times 100 \tag{16}$$

The reference compound is used to determine the amount of drug injected. To determine the BBB efflux clearance, 100–BEI% is calculated as

$$100 - \text{BEI}\% = \left(\frac{\frac{\text{Amount of test compound in brain}}{\text{Amount of reference in brain}}}{\frac{\text{Amount of test compound injected}}{\text{Amount of reference injected}}} \right) \times 100 \tag{17}$$

Nonlinear regression analysis of 100 – BEI% against time gives the apparent elimination constant k_{el}. The efflux clearance is obtained by multiplying k_{el} by the distribution volume $V_{u,brain}$, determined using the brain slice method [59] according to

$$\text{CL}_{\text{efflux}} = k_{el} \times V_{u,\text{brain}} \tag{18}$$

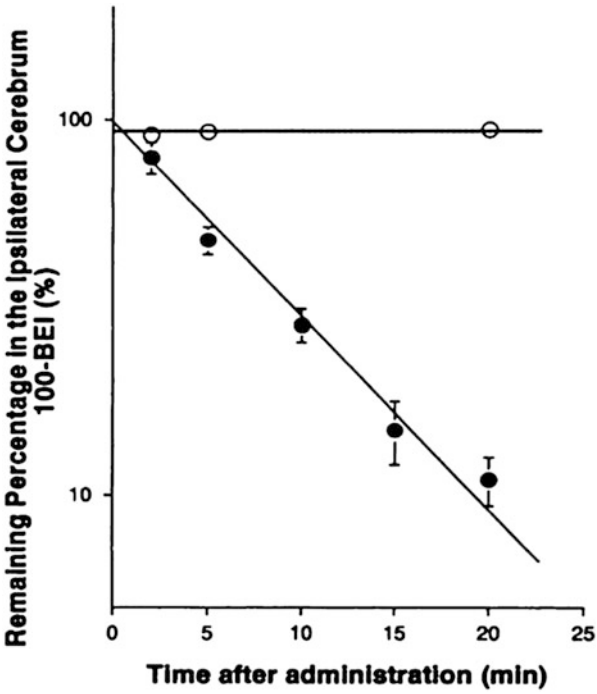

Fig. 6 Time course of BBB efflux clearance using 100–BEI (%) (see Eq. (16)) for [^{3}H]3-O-methyl-D-glucose (*closed circles*) and [^{3}H]L-glucose (*open circles*) in the ipsilateral cerebrum after intracerebral microinjection into the PAR2 region of rats. The reference compound was [^{14}C]inulin. From Kakee et al. [59] with permission from the publisher

3.5 *Percentage of the Injected Dose*

The percentage of a systemically injected dose (ID) that is delivered to the brain provides an estimate of the extent of drug transport into the brain. The percentage of the dose at time t after administration can be determined from the PA and the area under the curve of the plasma concentrations between times 0 and t (AUC) [52]:

$$\%\mathrm{ID/g\_brain}_0^t = \mathrm{PA} \times \mathrm{AUC}_0^t \tag{19}$$

Thus, the amount of the dose that reaches the brain is dependent on the plasma pharmacokinetics and the permeability of the BBB to the drug (influx). However, the amount reaching the brain is also determined by the influx/efflux ratio according to Eqs. (11)–(13), making the estimation of PA in Eq. (19) erroneous if the times studied are not very short. The percentage of the dose reaching the brain is not usually compared with the AUC, but is calculated directly. The value of this method is questionable if other methods are available.

3.6 Microdialysis

Microdialysis has become a well-established technique in the field of neuroscience, mostly for measuring the concentrations of endogenous substances, but also a very important technique for measuring drug concentrations in the brain [25, 26, 62–66]. The specific property of microdialysis that sets it apart is that it maps the concentrations of the unbound compound in the tissue in which the probe is placed, making it possible to correlate concentrations with pharmacological responses and receptor binding.

Microdialysis is mainly used to measure the extent of transport, but can also be used to estimate influx and efflux clearances at the BBB by including $V_{u,brain}$ measurements and modeling the data [67, 68]. The advantages of microdialysis are that it can be used to sample the local concentrations of unbound drug and to sample multiple time-points within the same individual. This reduces the number of animals needed while at the same time improving the amount of detailed information. The major disadvantage of microdialysis for studying drug pharmacokinetics is that tubings and probes can adsorb the compound of interest, resulting in erroneous concentrations and time profiles. An in vitro check of the adsorption behavior is thus required before proceeding to in vivo studies. See Fig. 7 and Table 6.

The microdialysis probe is placed in the selected area of the brain with the help of stereotactic coordinates. For BBB transport studies, a probe can also be placed in the jugular vein or regular blood can be sampled. The probe can be positioned during surgery on the day, or even several days, before the study is performed. Alternatively, a guide cannula can be surgically positioned on the day of surgery and the probe inserted just before the experiment. In some studies, the probe is inserted in anesthetized animals just before the experiment starts; however, this can cause leakage and disturbances in BBB function. On the other hand, waiting for too long (>3–5 days) after insertion of the probe can result in an inflammatory response which may hamper exchange across the probe [69, 70]. Nonetheless, this does not seem to be too influential when studying exogenous compounds.

In vivo recovery estimations are needed for quantitative studies when mapping the extracellular environment and BBB transport of drugs. Measurement of in vitro recovery can never adequately replace in vivo estimations, as the tissue surrounding the probe significantly influences the exchange across the probe membrane. These peri-probe processes include the exchange between extracellular sites and the vasculature, where active efflux transporters like P-gp have a substantial influence on the recovery, as well as the diffusion and metabolism, of the compound within the tissue [62]. In general, all processes that increase the turnover of the compound will increase its recovery.

Alternative methods of estimating the recovery of a drug in these studies include retrodialysis by drug or by calibrator [71], the no-net-flux method, and the dynamic no-net-flux method [72]. A deuterated version of the compound to be studied is the best choice for the calibrator used in retrodialysis, as recovery can then be mapped

Fig. 7 A cartoon of a microdialysis probe on a cast of the capillary network in the brain, showing the semipermeable membrane where exchange between the brain ISF and the dialysis fluid takes place. The picture can be found at http://www.leidenuniv.nl/en/researcharchive/index.php3-c=205.htm

Table 6 Advantages and disadvantages of microdialysis for studying BBB drug transport

Advantages	Disadvantages
Measures unbound drug concentrations	Possible tissue damage during probe implantation
Continuous measurements possible for relatively long periods	In vivo recovery measurements necessary for quantitative measurements (validation crucial)
Any tissue can be studied	The analyte has to be suitable for dialysis (to prevent drug adsorption to probe and tubing material)
Different sites can be measured simultaneously	No access to the intracellular biophase (although no method can accomplish this yet)
Gives detailed, thorough information	Slow
Because crossover studies are possible in small animals, fewer animals are needed	Necessary to adapt the experimental design to methodological aspects, including analytical sensitivity vs collection interval and flow rate
No loss of body fluids (blood, CSF, etc.)	
Possible to administer test compounds locally	
Low variability because fewer animals required	
No vascular contamination of tissue samples	
No sample cleanup needed	
Can correlate drug concentrations with effects in the same tissue	

throughout the study [73]. This requires liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. Alternatively, a compound that is closely related chemically can be used, with the caveat that it may be treated differently by transporters at the BBB, thus resulting in a different recovery than the drug of interest. This is especially important if drug interactions are studied, as the extent of changes in in vivo recovery of the study compound as a result of the interaction may differ from that of the calibrator. The compound of interest could also be studied before it is administered, to check its retrodialysis recovery. A washout period is then needed before the compound can be administered. However, changes during the study itself are then not mapped. This method cannot be used if the studied compound is endogenous.

With the microdialysis method, there is always a trade-off between the sampling duration wanted, the flow rate, and the analytical sensitivity. The lower the flow rate, the higher the recovery, but the smaller the recovered volumes for analysis, given a certain time-interval for sampling. A flow rate of 0.3 μL min^{-1} is used in clinical microdialysis studies, while preclinical studies use 0.5–2 μL min^{-1} or even higher. It may be advantageous to administer drugs as constant-rate infusions to reach steady state in order to counteract these problems. For retrodialysis recovery estimations, a lower recovery is often associated with a greater likelihood of error, as the concentrations of drug in the inflow of perfusate and the outflow of dialysate will be similar, in addition to the inherent variability in the chemical analysis [71].

An in vitro check of adsorption to the tubing should always be made before the in vivo studies [66]. This can be done as a first step, using various types of tubing and sampling every 10 min for 1 h, before adding the probe. Loss to and gain from the tubing are checked by adding the compound of interest to a test tube surrounding the probe and to the perfusate through the probe in sequence, with only blank buffers added between. The percentage loss and gain should be very similar if the data from the study are to be trusted, and there should be a "square-wave" curve when the solutions are changed, indicating that drug recovery does not lag behind as a result of slow release from the tubing and/or the probe. Addition of 0.5% albumin to the perfusate may help prevent the drug from sticking to tubings and the probe membrane, but this will not work if the drug has a high albumin-binding propensity (unpublished observations). Alternatively, the tubing and probe can be coated with a poloxamer such as Pluronic [74].

3.7 CSF Sampling

The CSF offers an accessible sampling site that has been used for many years to estimate the concentration of drugs in the brain. It is important to note that the CSF is a compartment on its own, with the blood–cerebrospinal fluid barrier (BCSFB) at the choroid plexus forming the interface with the blood. About 10% of the CSF is

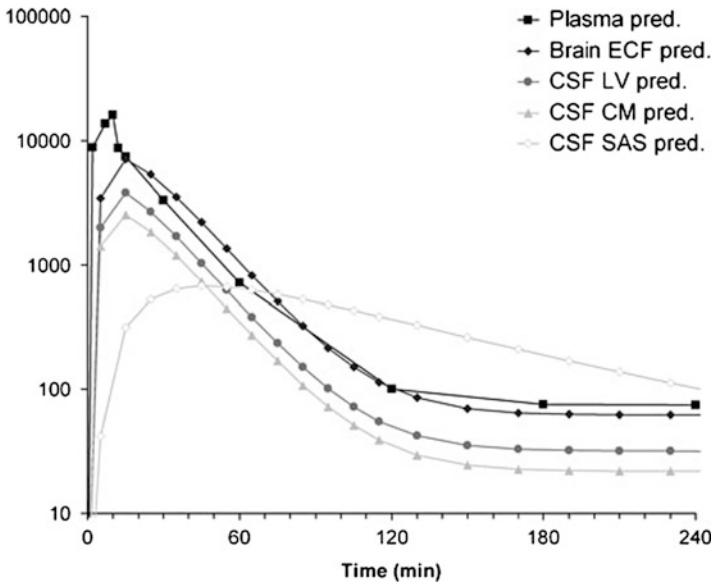

Fig. 8 Predicted concentrations of acetaminophen in plasma and various locations in the brain (*ECF* extracellular fluid) and CSF (*LV* lateral ventricle, *CM* cisterna magna, *SAS* subarachnoidal space), showing the delay in reaching peak concentrations in the CSF vs plasma, with the longest delay in the SAS. From Westerhout et al. [76] with permission from the publisher

made up from the bulk flow of fluid from the brain. The rest is produced at the choroid plexus.

The expression of transporters at the BCSFB may or may not be similar to that at the BBB. It has been shown in rats, for example, that the expression of P-gp is lower at the BCSFB than at the BBB, but that of multidrug resistance protein 1 (Mrp1) is significantly higher [75]. The introduction of microdialysis and other current methods has allowed the comparison of unbound drug concentrations between the brain and the CSF. As suggested by the differing expression of transporters [75], CSF concentrations often overpredict unbound drug concentrations in the brain if the drug is actively effluxed at the BBB and underpredict brain concentrations if the drug is actively taken up at the BBB [35].

Equilibration between the CSF and blood appears to be somewhat slower than that between the brain ISF and blood, as shown in Fig. 8 [76]. The profiles may differ between humans and rats, because the site of sampling differs: the subarachnoidal space (SAS) in humans and the cisterna magna in rats. Westerhout et al. developed an elegant model regarding the correlation of rat and human concentrations [76].

Sampling the CSF from the cisterna magna can be achieved either as a single sample or via a permanent cannula. After sampling, the volume of the CSF is decreased, and this could affect equilibration across the BCSFB. Therefore, the number of serial samples taken should be limited so as not to influence the equilibrium. Microdialysis in the ventricle is an alternative [76].

Table 7 Advantages and disadvantages of CSF sampling for studying BBB drug transport

Advantages	Disadvantages
CSF concentrations of unbound drug are closer to those in the brain than plasma concentrations	Different transporter expression in the BCSFB than at the BBB
Samples can also be obtained from humans	Different sampling sites in rodents and humans make comparisons difficult
	The possible increase in protein content in the CSF in some disease states can lead to erroneous estimations of drug concentrations for drugs that have high protein binding if this is not compensated for

If the differences between the CSF and brain parenchymal concentrations of drugs are taken into consideration, the CSF is an accessible surrogate site that gives an indication of the concentration range that can be expected in the brain (Table 7) [35, 77–80].

3.8 Positron Emission Tomography

Positron emission tomography (PET) can be used to measure both the rate and the extent of transport across the BBB. This method has the great advantage in that it is a noninvasive method that can be used in humans and can be used to study disease states [81]. Measuring BBB transport with PET requires both blood sampling and PET images of brain concentrations; this method is not used as commonly as some of the others [82]. The compound of interest is usually labeled with [^{11}C] and the total radioactivity is measured. The decay half-life ($t½$) of the tracer limits the time frame of the study to about three half-lives, which is about one hour for [^{11}C] ($t½ = 20$ min) and is about 5 h for [^{18} F] ($t½ = 110$ min).

It is necessary to subtract the radioactivity signals from the metabolites when using PET. It is highly likely that the metabolite-to-parent drug ratio in the brain differs from that in the plasma. The different ratios in the brain may be due to differences in nonspecific binding to brain parenchyma or in the extent of BBB transport of the drug and its metabolites (Table 8) [53, 82].

3.9 Combinatorial Mapping of $\mathbf{K}_{p,uu,brain}$

The partition coefficient ($K_{p,brain}$) can be determined by sampling whole brain tissue and plasma (Eq. (14)). Normally, a single dose is administered systemically, and brain and plasma samples are taken at one or several time-points. It is, of course, important that equilibrium has been attained between brain and plasma. The notion of

Table 8 Advantages and disadvantages of PET for studying BBB transport

Advantages	Disadvantages
Noninvasive	Expensive
Can be used in humans	Not all molecules can be labeled with a radioactive atom
Possible to obtain local information from specific brain sites	High technical challenges in equipment and data handling
	Measures total radioactivity

cassette (multiple) dosing has been studied to determine whether the administration of several compounds simultaneously works as well as individual administration, in order to save animals [83]. The results showed that interactions at the BBB between several compounds administered simultaneously are unlikely at the low doses used (1–3 mg kg^{-1}).

A *combinatorial map of* $K_{p,uu,brain}$ can be made using $K_{p,brain}$ determined as above, and $V_{u,brain}$ (or $f_{u,brain}$) and $f_{u,plasma}$ measurements [34]. Equation (14), or a modification that includes $1/V_{u,brain}$ instead of $f_{u,brain}$ (Eq. (20)), can then be used to calculate $K_{p,uu,brain}$. $K_{p,uu,brain}$ can also be determined using microdialysis [36]; however, the time needed for this method and the lack of its success with many lipophilic compounds mean that microdialysis is less feasible in a drug discovery setting.

Two parameters are used to measure the binding of drugs to the brain parenchyma. These are the $f_{u,brain}$, which is determined from equilibrium dialysis of diluted brain homogenate, and the volume of distribution of unbound drug in the brain, $V_{u,brain}$, which is determined from fresh brain slice measurements. These two parameters are related according to

$$f_{u,brain} \approx {}^{1}/_{V_{u,brain}} \tag{20}$$

The parameters describe the intra-brain distribution of the compounds studied, rather than the actual BBB transport of the compounds. Either one of the parameters are then used to obtain $K_{p,uu,brain}$. As discussed below, Eq. (20) should be used with caution as it is not always appropriate.

$V_{u,brain}$, which describes the average nonspecific binding to brain tissue, can be determined using the *brain slice technique* (Eq. (21)) [34, 59, 84, 85]. Fresh rat brain is sectioned into six 300 μm slices and put into a buffer. The proportion of buffer for specific slice weights is crucial for optimal equilibration [86]. The buffer is gently stirred at 37°C for 5 h. One slice is then used to measure viability, while the other five slices are used to determine the total brain concentrations. The buffer is sampled as a measure of the unbound drug ISF concentration:

$$V_{u,brain} = \frac{Q_{slice}}{C_{buffer}} \tag{21}$$

Table 9 Advantages and disadvantages of the combinatory mapping of $K_{p,uu}$ for studying BBB drug transport

Advantages	Disadvantages
Rapid	The combination of three measurements increases uncertainty
Can obtain $K_{p,uu}$ without the need for microdialysis, which is otherwise the only alternative	

where Q_{slice} is the amount of compound per gram of brain slice and C_{buffer} is the concentration of compound in the surrounding buffer, which is assumed to be equal to the brain ISF concentration, using units of mL g brain^{-1}. Values above unity indicate binding to brain parenchymal cells and values below unity indicate restricted distribution of the compound into brain parenchymal cells. If cassette dosing is used, the combined concentration of the compounds should be 1 μM at most [34]. A detailed protocol for the brain slice procedure has been published by Loryan et al. [85].

The *brain homogenate method* is used to determine $f_{u,brain}$ [87, 88]. A homogenate of the brain tissue is mixed with 2–9 volumes of buffer and is dialyzed across a semipermeable membrane against buffer until equilibrium is reached. Frozen brain homogenate can be used. The disadvantage of this method is that the parenchymal cells are destroyed during homogenization, and differences in pH between subcellular structures are subsequently lost. When the brain slice and brain homogenate methods were compared, it was seen that the brain homogenate results required recalculation using the pH partitioning model to better estimate the binding and intracellular partitioning of the drug [51]. Unpublished observations show that results may differ between the two methods even when pH partitioning is taken into account. Di et al. have shown that binding to brain tissue homogenate is very similar between species (Table 9) [89].

4 Conclusions

There are currently several methods available for studying the rate and extent of drug transport across the BBB, both preclinically and clinically. It is important that the question to be answered correlates with the method used. Methods that measure the extent of delivery to the brain are more likely to give clinically relevant estimations of BBB penetration than those measuring the rate of transport.

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