Chapter 13
Drug Discovery Methods for Studying Brain Drug Delivery and Distribution

Irena Loryan and Margareta Hammarlund-Udenaes

Abstract Methods used in drug discovery laboratories for assessing the delivery of small molecules to the brain have changed significantly in recent years. There is now more focus on measuring or estimating target unbound drug concentrations in the brain and evaluating the quantitative aspects of drug transport across the blood-brain barrier (BBB). The techniques for the investigation of the rate and extent of BBB transport of new chemical entities (NCEs) are discussed in this chapter. Combinatory methodology for rapid mapping of the extent of brain drug delivery via assessment of the unbound drug brain partitioning coefficient is presented. The chapter also explains the procedures for approximation of subcellular distribution of NCEs, particularly into the lysosomes. The principles, technical issues, advantages, and potential applications of techniques for evaluation of intra-brain distribution, i.e., equilibrium dialysis-based brain homogenate and brain slice methods, are described. The assessment of the extent of BBB transport and intracellular distribution of NCEs, the identification of intra-brain distribution patterns, and their integration with pharmacodynamic measurements are valuable implements for candidate evaluation and selection in drug discovery and development.

Keywords Brain homogenate method · Brain slice method · Lysosomal trapping · $V_{u,brain}$ · Combinatory mapping approach · Translation

Abbreviations

A_{brain}	Amount of drug in brain tissue
$AUC_{tot,brain}$	Area under the total brain concentration-time curve
$AUC_{tot,plasma}$	Area under the total plasma concentration-time curve
BBB	Blood-brain barrier

I. Loryan (✉) · M. Hammarlund-Udenaes
Translational PKPD Research Group, Department of Pharmacy, Uppsala University, Uppsala, Sweden

SciLife Lab, Solna, Sweden
e-mail: Irena.loryan@farmaci.uu.se; mhu@farmaci.uu.se

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BCRP	Breast cancer resistance-associated protein
BCSFB	Blood-CSF barrier
CB	Cellular barrier
C_{buffer}	Concentration of compound in the buffer (brain slice method)
$C_{tot,blood}$	Total drug concentration in blood
CNS	Central nervous system
CSF	Cerebrospinal fluid
$C_{tot,plasma}$	Total drug concentration in plasma
$C_{tot,brain}$	Total drug concentration in brain
$C_{u,brainISF}$	Unbound drug concentration in brain interstitial fluid
$C_{u,cell}$	Unbound drug concentration in intracellular fluid
$C_{u,cyto}$	Unbound drug concentration in cytosol
$C_{u,lyso}$	Unbound drug concentration in lysosomes
$C_{u,plasma}$	Unbound drug concentration in plasma
DMPK	Drug metabolism and pharmacokinetics
ECF	Extracellular fluid (same as ISF)
ED	Equilibrium dialysis
ER	Efflux ratio
$f_{u,brain}$	Fraction of unbound drug in brain homogenate
$f_{u,brain,corrected}$	$f_{u,brain}$ corrected for pH partitioning into cells
$f_{u,hD}$	Fraction of unbound drug in diluted brain homogenate
$f_{u,plasma}$	Fraction of unbound drug in plasma
HTS	High-throughput screening
ICF	Intracellular fluid in the brain
ISF	Interstitial fluid in the brain
K_d	Equilibrium dissociation constant
$K_{p,brain}$	Ratio of total-brain-to-total plasma drug concentrations (also abbreviated as BB)
$K_{p,uu,brain}$	Ratio of brain ISF-to-plasma unbound drug concentrations
$K_{p,uu,cell}$	Ratio of brain ICF-to-ISF unbound drug concentrations
$K_{p,uu,cyto}$	Ratio of cytosolic-to-extracellular unbound drug concentrations
$K_{p,uu,lyso}$	Ratio of lysosomic-to-cytosolic unbound drug concentrations
LC-MS/MS	Liquid chromatography tandem mass spectrometry
logBB	Logarithm of $K_{p,brain}$ (BB)
MWCO	Molecular weight cut-off
NCE	New chemical entity
neuroPK	Neuropharmacokinetics
P_{app}	Unidirectional apparent permeability coefficient measured in the apical-to-basolateral direction (cm/s)
PBS	Phosphate-buffered saline
PD	Pharmacodynamics
PET	Positron emission tomography
P-gp	P-glycoprotein
PK	Pharmacokinetics
PLD	Drug-induced phospholipidosis

PS	Permeability surface area product (μL/min · g brain^{-1})
V_{ss}	Apparent volume of distribution at steady state
$V_{u,brain}$	Volume of distribution of unbound drug in brain (mL · g brain^{-1})

13.1 Introduction

The existing situation in the discovery and development of drugs for CNS-related conditions is unprecedentedly desperate, in the face of enormous unmet medical need (Eaton et al. 2008; Schoepp 2011; Schwab and Buchli 2012; Butlen-Ducuing et al. 2016; Cummings et al. 2016). The probability of success with emerging breakthrough first-in-class CNS drugs is small. Further, because neurotherapeutic drugs move more slowly in the development pipeline (compared to, e.g., AIDS antivirals), they require a relatively extended time to get to the market (Kaitin and DiMasi 2011). Despite immense efforts from the drug industry and academia, it could be thought that CNS drug discovery is currently almost in a blind alley. In contrast, however, Weaver and Weaver have used molecular modeling to reach the conclusion that the pharmaceutical industry is still in its infancy when it comes to exploring the neuroactive chemical space (Weaver and Weaver 2011). In addition, multiple pharmaceutical companies are on the way of the development of various biologicals including antibodies for treatment of neurological diseases (Farrington et al. 2014; Freskgard and Urich 2017; Stanimirovic et al. 2018).

The reasons for the apparent failure of CNS drug discovery, such as lack of clinically translatable animal disease models, lack of relevant biomarkers, and inadequate exposure of the CNS to potential drugs because of the blood-brain barrier (BBB), are generally acknowledged and are challenging to resolve (Jeffrey and Summerfield 2007; Hammarlund-Udenaes et al. 2008; Neuwelt et al. 2008; Kelly 2009; Reichel 2009; Abbott et al. 2010; Brunner et al. 2012; Mehta et al. 2017).

This chapter is dedicated to the quantitative aspects of drug transport across the BBB and contemporary methods of assessing CNS exposure to NCEs in drug discovery and development programs. From drug discovery perspectives, it is important to mention that the BBB per se is not the only obstacle to drug delivery to the brain. Inadequate understanding of the principles of drug transport at the BBB and a lack of appropriate interpretation of target exposure could also be seen as hindrances to progression (Hammarlund-Udenaes et al. 2009).

As explained in the article by Elebring and colleagues, it is becoming more and more imperative to separate and define two crucial aspects of drug discovery: efficacy (i.e., doing the right things) and efficiency (i.e., doing things right) (Elebring et al. 2012). In the modern pharmaceutical industry, we often observe the problems associated with "high-throughput" thinking (high efficiency) which typically biases biopharmaceutical scientists toward simple "one-fits-all" solutions. Alternatively, a tailored specific approach could be more effective. If this approach is to be applied to brain drug delivery, it is important initially to define what is meant by brain drug

delivery and subsequently to identify the relevant core neuropharmacokinetic (neuroPK) parameters and applicable methods for the assessment of CNS exposure.

Because the novel strategies available for CNS drug delivery differ widely (invasive, noninvasive), the definitions of brain drug delivery, and consequently the choice of appropriate neuroPK parameters, are also divergent (Pardridge et al. 1992; Thorne et al. 1995; Begley 1996, 2004; Huwyler et al. 1996; Pardridge 1997; Li et al. 1999; Scherrmann 2002; Reichel et al. 2003; Garberg et al. 2005; Garcia-Garcia et al. 2005; Terasaki and Ohtsuki 2005; Pardridge 2006; de Boer and Gaillard 2007; Hammarlund-Udenaes et al. 2008; Wang et al. 2009; Gaillard et al. 2012; Stevens et al. 2012; de Lange 2013a). This chapter focuses on "classical" blood-to-brain delivery of small molecules, where drug delivery from the blood to the brain through the BBB can be described by *rate* and *extent* parameters (see Chap. 7, which discusses the pharmacokinetic concepts of brain drug delivery).

The *rate* of BBB transport is commonly characterized by the **permeability surface area product** (PS, mL/min/kg body weight). Being unidirectional, the PS describes the speed at which the drug enters the brain (Fenstermacher 1992; Tanaka and Mizojiri 1999; Gaillard and de Boer 2000; Summerfield et al. 2007; Liu et al. 2008; Zhao et al. 2009). Generally, fast permeation is a key requirement for drugs when rapid CNS onset is wanted, e.g., for general anesthetics and analgesics. Although only a limited number of compounds in a few pharmacological classes are required to permeate the brain quickly, the apparent BBB permeability (P_{app}; measured in vitro) is among the parameters considered by pharmaceutical industry to be essential for evaluation of BBB penetration in drug development programs (Liu et al. 2005; Jeffrey and Summerfield 2007; Summerfield et al. 2007). Moreover, combined with an in vitro P-glycoprotein (P-gp) assay, it is used as a basis for guiding the lead optimization and candidate selection (Di et al. 2012a). To make this point more explicit, it is worth mentioning that permeability-limited drug distribution in the brain (<10% of cerebral blood flow or logPS < −2.9) is a very rare phenomenon associated with a slow equilibration time in the brain and is not a matter of concern for potential CNS drugs intended for chronic administration (Abraham 2011; Kell et al. 2011, 2013; Deo et al. 2013). It is obvious that permeability as a test for BBB penetration is overpromoted in the pharmaceutical industry. The methods used for permeability measurements are not covered in this chapter, but are thoroughly discussed in Chaps. 7 and 8.

In the drug discovery setting, the *extent* of BBB transport is traditionally evaluated in rodents using the steady-state ratio of total-brain-to-total-plasma drug concentrations ($K_{p,brain}$, BB, or logBB). Many generations of CNS drug discovery programs have been driven by optimizing $K_{p,brain}$, which has led to mass production of CNS compounds with high lipophilicity and development of the phenomenon known as the "lipidization trap": higher lipophilicity-higher $K_{p,brain}$ value-higher brain tissue binding-lower fraction of unbound drug in the brain (Deo et al. 2013). Because it is affected by nonspecific binding of the drug to plasma proteins and brain tissue, $K_{p,brain}$ masks the actual BBB net flux value (Lin et al. 1982; Lin and Lin 1990; Kalvass and Maurer 2002; Summerfield et al. 2007; Wan et al. 2007; Hammarlund-Udenaes et al. 2008; Read and Braggio 2010; Friden et al. 2011;

Longhi et al. 2011). The use of $K_{p,brain}$ for optimizing novel neurotherapeutics has thus created further confusion in the field. In this regard, the steady-state **ratio of brain interstitial fluid (ISF) to plasma unbound drug concentrations ($K_{p,uu,brain}$)** is currently considered to be the most relevant measure of BBB function (Gupta et al. 2006; Jeffrey and Summerfield 2007; Hammarlund-Udenaes et al. 2008, 2009; Liu et al. 2009b; Reichel 2009; Read and Braggio 2010; Di et al. 2012a; Doran et al. 2012; Loryan et al. 2014, 2016; Schou et al. 2015).

$K_{p,uu,brain}$, the unbound drug brain partitioning coefficient, allows the assessment of the concentration of cerebral unbound drug, which is the main pharmacokinetic determinant of CNS activity of neurotherapeutics, based on a given plasma concentration (Harashima et al. 1984; Gupta et al. 2006; Kalvass et al. 2007a; Liu et al. 2009b; Watson et al. 2009; Hammarlund-Udenaes 2010; Bundgaard et al. 2012b). Thus far, cerebral microdialysis has been the "gold" standard for the measurement of unbound cerebral concentrations in the brains of animals and humans (Elmquist and Sawchuk 1997, 2000; Hammarlund-Udenaes et al. 1997; de Lange et al. 1999; Kitamura et al. 2016; Hammarlund-Udenaes 2017). However, the practice of microdialysis for evaluation of BBB penetration in a drug discovery setup is limited mainly due to extensive adsorption to plastic tubing and probe. Nevertheless, a clinically relevant picture of the extent of brain drug delivery can be achieved using the combinatory mapping approach (CMA, Fig. 13.1) by means of evaluation of

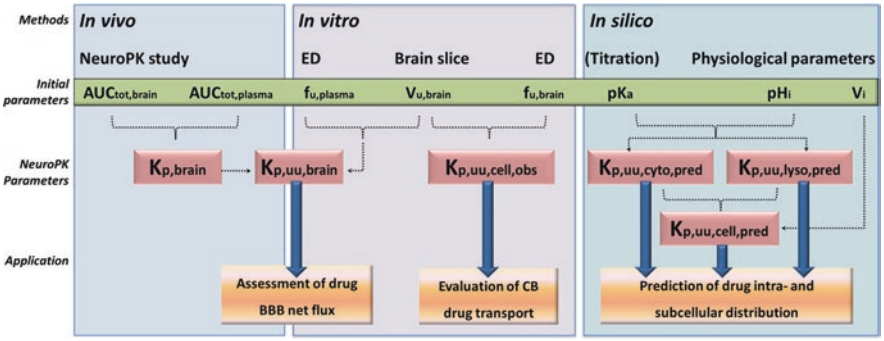

Fig. 13.1 An illustration of the combinatory mapping approach (CMA) in the form of a screening toolbox for the evaluation of unbound drug CNS exposure required for the selection of novel drug candidates. Figure obtained from Loryan et al. (2014). The platform comprising of in vivo, in vitro, and in silico toolboxes. Total drug brain and plasma exposure (e.g., by means of area under the curve of concentration-time profiles, $AUC_{tot,brain}$, and $AUC_{tot,plasma}$) determined in an in vivo neuroPK study is essential for the assessment of the brain partitioning coefficient $K_{p,brain}$. In vitro measurements of drug plasma and brain tissue binding properties using equilibrium dialysis (ED) and brain slice techniques are required for the estimation of $K_{p,uu,brain}$ and $K_{p,uu,cell}$ neuroPK parameters. Compound-specific pKa values in combination with the physiological estimates of pH (pHi) of the relevant compartments (i = plasma, interstitial fluid, cytosol, or lysosomes) are used for in silico calculation of drug subcellular distribution, i.e., $K_{p,uu,cyto,pred}$ and $K_{p,uu,lyso,pred}$. Physiological volumes (Vi) of interstitial fluid, cytosol, and lysosomes with $K_{p,uu,cyto,pred}$ and $K_{p,uu,lyso,pred}$ are used for the calculation of $K_{p,uu,cell,pred}$. Assessed neuroPK parameters in conjunction with relevant pharmacodynamics readouts are recommended to be used for evaluation and selection of novel drug candidates

pharmacokinetic (PK) parameters such as $K_{p,brain}$, the volume of distribution of unbound drug in the brain ($V_{u,brain}$), and the fraction of unbound drug in the plasma ($f_{u,plasma}$) (Chap. 7 and Sect. 13.5).

A very important element of brain drug disposition, although it is unrelated to the BBB, is the *intracerebral distribution* of the drug, which is discussed in Sects. 13.2, 13.3 and 13.4. Enhanced understanding of the distribution of the drug in the brain provides new perspectives on the pharmacodynamics of neurotherapeutics. Typically, brain tissue binding is measured as the **fraction of unbound drug in the brain** ($f_{u,brain}$) using an equilibrium dialysis (ED) technique to assess the extent of nonspecific binding to the brain tissue (Kalvass and Maurer 2002; Kalvass et al. 2007a; Wan et al. 2007; Friden et al. 2011; Longhi et al. 2011; Di et al. 2012b). The method is mainly assessing intracellular binding (Friden et al. 2007; 2011).

Alternatively, the **volume of distribution of unbound drug in the brain** ($V_{u,brain}$), estimated using the fresh brain slice method, can allow assessment of the overall uptake by the brain tissue (Kakee et al. 1997; Liu et al. 2006; Benkwitz et al. 2007; Friden et al. 2009a; Kodaira et al. 2011; Uchida et al. 2011a). In this chapter, we have chosen to express information from brain homogenate studies as $f_{u,brain}$ and information from brain slice studies as $V_{u,brain}$ to differentiate and clarify the information as much as possible. Both these parameters, $V_{u,brain}$ and $f_{u,brain}$, permit the estimation of the concentration of unbound drug in brain ISF ($C_{u,brainISF}$) using total brain concentration ($C_{tot,brain}$) measurements and give an indication of the probable extracellular target engagement. However, the intracellular concentration of unbound drug is also of great interest. In view of this, approximation of the **ratio of brain intracellular fluid (ICF) to ISF unbound drug concentrations** ($K_{p,uu,cell}$) may be beneficial for understanding the pharmacological query related to intracellular targets and may be strategically influential (Friden et al. 2007). The $K_{p,uu,cell}$ concept is innovative, as it provides the basis for an increased awareness of the impact of cellular barrier function on intracerebral drug distribution, which has hitherto been neglected in drug discovery programs.

The approaches applied for prediction, assessment, and optimization (Chap. 12) of the BBB transport of NCEs, such as in silico (Chap. 14), in vitro (Chaps. 8 and 9), and in vivo methods (Chaps. 10 and 11), depend on the development phase of the drug and the questions of interest.

13.2 The Brain Homogenate Method for $f_{u,brain}$

The concentration of unbound drug in the brain, estimated using $C_{tot,brain}$ corrected for brain tissue binding, is a surrogate for $C_{u,brainISF}$. $C_{u,brainISF}$ is currently considered to be the most relevant parameter for measuring the pharmacological response of neurotherapeutics (Bouw et al. 2001; Bostrom et al. 2006; Bundgaard et al. 2007, 2012b; Kalvass et al. 2007b; Liu et al. 2009b; Watson et al. 2009; Hammarlund-Udenaes 2010; Smith et al. 2010; Westerhout et al. 2011).

Brain tissue binding can be determined by various methods, including ED, stepwise ED, ultrafiltration, ultracentrifugation, gel filtration, and absorption by brain lipid membrane vesicles stabilized on silica beads (TRANSIL brain absorption kit) (Fichtl et al. 1991a; Kalvass and Maurer 2002; Mano et al. 2002; Vuignier et al. 2010; Longhi et al. 2011). This section focuses on the ED technique for the estimation of $f_{u,brain}$, which is presently used in drug discovery programs in a high-throughput manner.

13.2.1 Equilibrium Dialysis

In 2001, Kariv et al. presented the successful development of a 96-well equilibrium dialysis (ED) plate suitable for evaluation of plasma protein binding for large numbers of biologically active NCEs during high-throughput screening (HTS) (Kariv et al. 2001). Contemporary 96-well ED apparatus allows the researcher to examine a large number of samples, time points, or replicates in the same experiment.

Using a similar approach, Kalvass and Maurer introduced a high-throughput ED technique designed for the determination of brain tissue binding (Kalvass and Maurer 2002). The method rapidly became standard, and it is currently widely used for the estimation of $f_{u,brain}$ for a large number of chemically diverse compounds (Summerfield et al. 2006; Wan et al. 2007; Di et al. 2011; Friden et al. 2011; Longhi et al. 2011). The need for protein binding data in combination with the large number of compounds created from combinatorial chemistry has stimulated the development of a novel cassette-based pooling approach which allows simultaneous assessment of $f_{u,brain}$ or $f_{u,plasma}$ for more than five compounds per sample (Fung et al. 2003; Wan et al. 2007; Plise et al. 2010; Longhi et al. 2011).

Several research groups and pharmaceutical companies have validated the compatibility of the high-throughput ED techniques (96-, 48-well formats) with most standard laboratory supplies and robotics (Banker et al. 2003; van Liempd et al. 2011). Several devices based on a 96-well format are currently on the market (e.g., the Equilibrium Dialyzer-96 from Harvard Biosciences (Holliston, MA, USA), the Rapid Equilibrium Device from Thermo Scientific/Pierce (Rockford, IL, USA), and the Micro-Equilibrium Dialysis Device from HTdialysis LLC (Gales Ferry, CT, USA)).

13.2.1.1 Principles

The semipermeable membrane between the buffer and the homogenate compartments in the ED apparatus acts as a molecular filter permitting diffusion against the concentration gradient of molecules smaller than a definite molecular weight. The drug (1–5 μM) is added to the brain homogenate (donor side) and is sampled from both the donor and the buffer (receiver) sides. To be able to perform ED, the brain homogenate needs to be diluted with phosphate-buffered saline (PBS; Sect.

13.2.1.2.2), commonly with dilution factors of either three (Kalvass and Maurer 2002) or five (Di et al. 2012b). As a general rule, the drug-tissue protein interaction is reversible, and, in the majority of cases, equilibrium rapidly occurs between the unbound and bound molecular species. At equilibrium, the unbound fraction in diluted brain homogenate can be calculated as

$$f_{u,hD} = \frac{C_{receiver}}{C_{donor}} \tag{13.1}$$

where $f_{u,hD}$ is the measured experimental fraction of unbound compound in diluted (D) brain homogenate, $C_{receiver}$ is the concentration of the compound in the buffer, and C_{donor} is the concentration of compound in the donor chamber at equilibrium.

The interaction between the compound/drug and brain tissue is, in most cases, a rapid and reversible process governed by the law of mass action, given that binding does not alter the drug or protein (Klotz 1973). The model assumes that binding between drug and brain tissue takes place in a single step and that the drug interacts with only one binding site on the protein. The equilibrium is described as

$$[D]+[B] \leftrightarrow [DB] \tag{13.2}$$

where [D] and [B] represent the unbound drug and brain tissue protein concentrations and [DB] represents the concentration of the drug-brain tissue protein complex.

The equilibrium dissociation constant (K_d) characterizes the concentration of unbound drug that occupies half of the binding sites on the protein at equilibrium:

$$K_d = \frac{k_{off}}{k_{on}} = \frac{[D][B]}{[DB]} \tag{13.3}$$

Accordingly, the fraction of unbound drug can be described as

$$f_{u,hD} = \frac{[DB]}{[B]+[DB]} = \frac{K_d}{[B]+K_d} \tag{13.4}$$

Rearranging Eq. 13.4 gives

$$K_d = \frac{f_{u,hD}[B]}{1-f_{u,hD}} \tag{13.5}$$

The unbound drug fraction usually increases as the brain homogenate is diluted. Therefore, $f_{u,hD}$ in the brain homogenate has to be corrected for dilution (Kurz and Fichtl 1983). There are several issues related to the dilution of the brain homogenate and subsequent adjustment methods (Fichtl et al. 1991b). The relationship between

the measured unbound drug fraction and the dilution factor is typically not linear (Kurz and Fichtl 1983). The relative impact of dilution of the brain homogenate on the formation of drug-brain tissue protein complexes has been thoroughly discussed by Romer and Bickel (Romer and Bickel 1979). Assuming two different concentrations of brain tissue binding components $[B]_1$ and $[B]_2$ with unbound drug fractions $f_{u,brain}$ and $f_{u,hD}$, Eq. 13.5 can be rewritten as

$$1 = \frac{f_{u,brain} \cdot [B]_1 \cdot \left(1 - f_{u,hD}\right)}{f_{u,hD} \cdot [B]_2 \cdot \left(1 - f_{u,brain}\right)} \tag{13.6}$$

The ratio of $[B]_1/[B]_2$ is projected as the brain homogenate dilution factor D. Hence, Eq. 13.6 can be reorganized to obtain the fraction of unbound drug in the undiluted brain tissue homogenate, which is used to calculate $f_{u,brain}$:

$$f_{u,brain} = \frac{\frac{1}{D}}{\left(\left(\frac{1}{f_{u,hD}}\right) - 1\right) + \frac{1}{D}} \tag{13.7}$$

13.2.1.2 Technical Challenges

The implementation of a 96-well ED plate improved the robustness of the ED method and allowed the use of volumes of brain homogenate and/or plasma as small as 30 µl (e.g., HTdialysis LLC). Although ED is regarded as a "gold" standard method, it has drawbacks which need to be discussed along with the advantages of the method. The equilibration time, concentration of drugs and proteins, membrane surface area, membrane features, and molecular charges can all crucially affect the rate of dialysis.

Selection of Dialysis Membrane

Dialysis membranes consist of a spongy matrix of cross-linked polymers with different pore ratings or molecular weight cut-off (MWCO) points. The MWCO is defined by the molecular weight of solute that is 90% retained by the membrane during a 17-h period. Various membranes (e.g., cellulose ester, regenerated cellulose, and polyvinylidene difluoride) with a range of MWCOs from 3.5K to 50K are applicable for ED. The most commonly used MWCO range is 12–14K.

A potential caveat of the ED method is the risk of nonspecific adsorption of drugs or proteins onto the chamber walls and the dialysis membrane (Vuignier et al. 2010). The use of an inert reusable 96-well Teflon construction minimizes

nonspecific binding of test compounds to the apparatus. However, the investigation of different types of dialysis membranes could be beneficial for the selection of the most suitable material.

Recovery (also called mass balance) is traditionally evaluated to account for nonspecific binding and is used as an acceptance criterion for ED-based experiments. However, a recent investigation found that recovery had no influence on $f_{u,brain}$ or $f_{u,plasma}$ (Di et al. 2012b). These researchers recommended focusing on stability issues as a main cause of uncertainty in the binding experiments instead.

Preparation of Brain Homogenate

Because an undiluted brain tissue homogenate is paste-like in consistency and difficult to handle, it is diluted with PBS pH7.4. However, this raises several questions concerning the trustworthy conversion of the brain tissue binding values estimated from diluted homogenate into values for the original protein concentrations in the brain tissue. The dilution factor may not affect the final $f_{u,brain}$ measurement (unpublished observations), and various dilution factors have been used. For example, Kalvass and colleagues diluted with two volumes of PBS (Kalvass and Maurer 2002; Liu et al. 2005; Friden et al. 2007; Summerfield et al. 2007; Wan et al. 2007), while Di and co-authors diluted with four volumes of Dulbecco's PBS (Di et al. 2011).

Either frozen or fresh brain tissue can be used to prepare the brain homogenate. However, because of limited supplies of fresh brain homogenate, frozen brain homogenate is often used in drug discovery programs (Di et al. 2012b). To date, no systematic study has been carried out to confirm or reject the existence of differences in brain tissue binding measured using fresh and frozen brain homogenates.

Depending on the method of exsanguination, brain tissue may contain some serum albumin as a result of the residual blood left in the tissue (Glees and Voth 1988). The presence of residual blood in the brain homogenate could affect $f_{u,brain}$ measurement, predominantly for compounds with high affinity for serum albumin (Longhi et al. 2011). Friden and co-workers demonstrated that the procedure of exsanguination of the animal before sampling the brain tissue could influence the residual volume of blood in the brain (Sect. 13.6) (Friden et al. 2010). Thus, the method of sacrificing animals should be standardized with the aim of reducing the residual volume of blood in the brain tissue. As a precautionary action, intracardial perfusion with cold phosphate-buffered saline (PBS) before extraction of the brain could be useful (Longhi et al. 2011). It has been proposed that determination of the serum albumin and total protein content in a brain tissue homogenate could aid the characterization and normalization of different batches (Kodaira et al. 2011; Longhi et al. 2011).

Equilibration Process

After spiking the diluted brain homogenate with the compound(s) of interest, usually up to 150 μL, aliquots are usually loaded into the 96-well ED apparatus and dialyzed against an equal volume of PBS. Compounds with poor aqueous solubility are typically considered to be problematic and limit the use of ED. Equilibrium is generally achieved by incubating the 96-well ED apparatus in a 37°C incubator at 155 rpm for 4–6 h (Kalvass and Maurer 2002). However, if more exact information is wanted, it could be an advantage to perform an initial set of studies to determine the time required for the system to reach equilibrium, as slow drug-protein dissociation may occur.

The equilibration time needed in ED, normally 4–6 h, is considered to be one of the drawbacks of the method if the compounds studied are unstable in the plasma or brain homogenate. Moreover, the equilibration time is associated with a volume shift that takes place because of the semipermeable membrane and the presence of proteins. This volume shift can be as large as 10–30% for ED with plasma (Huang 1983). Measuring drug concentrations on both sides of the membrane is therefore required.

Bioanalysis

During the equilibration period, the buffer side becomes enriched with ions, amino acids, lipids, carbohydrates, and any other molecules smaller than the MWCO of the dialysis membrane that are not already present in the buffer. The brain homogenate composition also changes as a result of osmotic pressure. The modifications in the composition of the buffer and brain homogenate could result in a "matrix" effect during subsequent liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis (e.g., ion suppression, enhancement of analyte signal) (Van Eeckhaut et al. 2009). Mixed-matrix and semi-automated mixed-matrix methods are currently being developed to decrease mass spectrometer run times and reduce the probability of experimental artifacts (Plise et al. 2010). For semi-automated mixed-matrix methods with a cassette-based approach, a single matrix is prepared following dialysis by mixing dialyzed plasma and buffer containing different test compounds from the same dialysis plate. The method should eliminate the need for standard curves, and increase the consistency of the sample matrix for LC-MS/MS analysis. This approach could easily be adopted when running the ED-based brain homogenate method and can be considered as a step toward further optimization of ED.

In conclusion, ED-based determination of $f_{u,brain}$ can be considered a proficient method. However, the biological and pharmacological meaning of the obtained values must be critically evaluated in relation to other neuroPK parameters (Sects. 13.4 and 13.5).

Recently, ED measures of the unbound fraction of drugs in plasma and brain were used as additional parameters for the interpretation of in vivo positron

emission tomography (PET) results, particularly for the estimation of unbound drug concentrations in the CNS and accurate quantification of receptor binding (Gunn et al. 2012).

13.3 The Brain Slice Method for $V_{u,brain}$

With respect to assessing the intracerebral distribution of small drug molecules, the ED-based brain homogenate method has drawbacks that are primarily linked to the disruption of brain parenchymal cells (Becker and Liu 2006; Liu et al. 2006; Friden et al. 2007, 2011). In this regard, the brain slice method is an advanced, well-functioning approach to the evaluation of the overall uptake of drugs into the brain tissue via determination of the volume of distribution of unbound drug in the brain ($V_{u,brain}$; mL · g brain^{-1}). This method has the benefits of being used in a regulated in vitro environment, while at the same time, preserving much of the cellular complex integrity, including cellular barriers and circuitry, and as a result conserving the functionality of the in vivo brain. As a result, the technique delivers information that is directly relevant to issues such as nonspecific binding to tissues, lysosomal trapping (Sect. 13.4.3), and active uptake into the cells.

The brain slice method was implemented by Henry McIlwain more than six decades ago and is nowadays widely used in neurobiology, electrophysiology, and quantitative neuropharmacology (McIlwain 1951b; Collingridge 1995). The first use of this method for evaluation of intracerebral distribution of substances aimed to estimate the uptake of nutrients such as glucose and amino acids into the brain (McIlwain 1951a; Blasberg et al. 1970; Newman et al. 1988a, 1991; Smith 1991). Later, the method was proposed for in vitro investigation of the distribution of drugs in the brain (Van Peer et al. 1981; Kakee et al. 1996, 1997; Ooie et al. 1997). There have been several efforts to establish mechanistic pharmacokinetic/pharmacodynamics links using brain slice methodology, e.g., for propofol (Gredell et al. 2004), etomidate (Benkwitz et al. 2007), and volatile agents (Chesney et al. 2003).

$V_{u,brain}$ can also be measured using cerebral microdialysis and total brain concentration measurements; this is currently accepted as an in vivo reference method for evaluating intracerebral drug distribution. When the fresh brain slice method was validated against microdialysis, $V_{u,brain}$ was within a threefold range of the microdialysis results for 14 of 15 investigated compounds (Friden et al. 2007). In contrast, when $V_{u,brain}$ was recalculated using data from the brain homogenate method for the same list of compounds, the results were less accurate. In particular, the brain homogenate method overpredicted in vivo $V_{u,brain}$ for compounds limited to intracerebral ISF distribution (e.g., morphine-3- and morphine-6-glucuronide, R- and S-cetirizine) and underpredicted the distribution of gabapentin, which has predominantly active cellular uptake (Friden et al. 2007). However, these results have been challenged. Liu and colleagues demonstrated that, for eight of the nine studied compounds (carbamazepine, citalopram, ganciclovir, metoclopramide, N-desmethylclozapine, quinidine, risperidone, 9-hydroxyrisperidone, and

thiopental), the $C_{u,brainISF}$ estimated using the brain homogenate method was within a threefold range of that obtained using cerebral microdialysis (Liu et al. 2009a). Nonetheless, these contrasting results should still be critically evaluated, since the microdialysis probes were calibrated using only in vitro recovery. Determination of $V_{u,brain}$ values that are more relevant to the in vivo situation, using fresh brain slices instead of brain homogenate, appears to be associated with more accurate assessment of $C_{u,brainISF}$ (i.e., $C_{u,buffer}$).

Despite the obvious benefits of the fresh brain slice method, it has not yet received wide acceptance in the drug industry compared to the brain homogenate method. The arguments against acceptance include that the method requires greater labor intensity. However, a high-throughput brain slice method has now been developed to fit the drug discovery format, thus offering new possibilities for the utilization of the method (Friden et al. 2009a; Loryan et al. 2013). Once the brain slice technique is established in a laboratory, one skilled assistant can perform up to four experiments per day. Up to ten compounds in one cassette can be tested simultaneously (prior consultation with an analytical chemist is obligatory). A series of three experiments is enough to obtain consistent results for one cassette. The detailed protocol of how to perform brain slice studies can be found in the publication by Loryan et al. (Loryan et al. 2013).

13.3.1 Section Heading 13.3.1

13.3.1.1 Principles

The use of the apparent $V_{u,brain}$, obtained in vivo using cerebral microdialysis (Eq. 13.8), to assess the distribution of drugs in the brain was first suggested by Wang and Welty (they used the abbreviation $V_{e,app}$) (Wang and Welty 1996). $V_{u,brain}$ describes the relationship between the total drug concentration in the brain and the unbound drug concentration in the brain ISF, regardless of BBB function.

Assessment of $V_{u,brain}$ using the in vitro fresh brain slice method is based on the assumption that *at equilibrium*, $C_{u,brainISF}$ is equal to the drug concentration in protein-free artificial extracellular fluid buffer (aECF). Thus, $V_{u,brain}$ (mL · g brain^{-1}) is calculated as the ratio of the amount of drug in the brain slice (A_{brain}, nanomoles · g brain^{-1}) to the measured final aECF after reaching equilibrium (C_{buffer}, micromoles · L^{-1}):

$$V_{u,brain} = \frac{A_{brain}}{C_{u,brainISF}} = \frac{A_{brain}}{C_{buffer}} \tag{13.8}$$

Because a certain volume of the aECF remains on the surface of the brain slice (V_i, mL · g slice^{-1}), even after removing the excess with filter paper, this has to be accounted for. V_i is estimated in a separate experiment using [^{14}C] inulin as described

in Friden et al. (2009a). Equation 13.8 is then rearranged to obtain $V_{u,brain}$ corrected for V_i $(1-V_i)$:

$$V_{u,brain} = \frac{A_{brain} - V_i \cdot C_{buffer}}{C_{buffer} \cdot (1 - V_i)} \tag{13.9}$$

As outlined by Wang and Welty, a $V_{u,brain}$ value that is higher than 1 mL · g brain^{-1} indicates intracellular accumulation or excessive brain tissue binding because it exceeds the total volume of water in the brain which is 0.8 mL · g brain^{-1} (Wang and Welty 1996). $V_{u,brain}$ values between 1 and 0.2 mL g brain^{-1} indicate limited distribution of drug in the brain ECF and ICF (Nicholson and Sykova 1998; Sykova and Nicholson 2008). As the volume of healthy adult rat brain ISF is 0.2 mL · g brain^{-1}, a volume below 0.2 mL · g brain^{-1} is not possible. However, it should be kept in mind that this technique does not account for possible intracerebral metabolism (Chap. 6).

In the literature, $V_{u,brain}$ is sometimes expressed as $f_{u,brain,slice}$ (Kodaira et al. 2011; Uchida et al. 2011a). It is important to keep in mind that $f_{u,brain,slice}$ could be considerably different from $f_{u,brain}$, as they obtained using different matrices, i.e., brain slice and brain tissue homogenate (Sect. 13.4).

13.3.1.2 Technical Challenges

Artificial Extracellular Fluid and Formation of Cassettes

It is important to preserve the viability of brain slices during the experiment and to mirror the in vivo cellular milieu as closely as possible. There are two main approaches to achieving this, regarding the medium used. One approach is based on the use of either fresh or thawed *plasma* as a medium for the incubation, with subsequent evaluation of the brain slice-to-plasma drug concentration ratio (Becker and Liu 2006). The second and more commonly applied approach is to use a *protein-free* artificial cerebrospinal fluid (CSF) or aECF as an incubation medium (Kakee et al. 1996, 1997). The latter simplifies the interpretations of the results obtained. A large number of formulations for aECF can be found in the literature (Newman et al. 1991; Kakee et al. 1996, 1997; Gredell et al. 2004; Friden et al. 2009a; Uchida et al. 2011a). In many of these, ascorbic acid is used as a natural free radical scavenger to protect cell membranes from lipid peroxidation and swelling of the brain slices (Rice 1999). The HEPES-buffered aECF containing 129 mM NaCl, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 25 mM HEPES, 10 mM glucose, and 0.4 mM ascorbic acid is a robust and practical formulation for sustaining the physiological pH (around 7.3 at 37 °C after 5-h incubation) for the high-throughput setup (Friden et al. 2009a).

Another critical requirement is an adequate oxygen supply. Either 100% humidified oxygen or carbogen (a mixture of 95% oxygen and 5% CO_2) can be used.

The brain slice method allows examination of up to ten compounds per experiment, covering a wide range of physicochemical properties and pharmacological targets, mixed together in the same *cassette* (the mixture of compounds under investigation is called the cassette) (Friden et al. 2009a; Kodaira et al. 2011). Low concentrations of compounds (e.g., 0.1–0.2 μM) are preferable. The summed concentration of all the drugs in the cassette should not exceed 1 μM (Friden et al. 2009a). Application of higher concentrations of various compounds can lead to accumulation of compounds in the acidic compartments of the cells (i.e., lysosomes) or competition for specific cell membrane transporters with subsequent incorrect values for $V_{u,brain}$. For instance, it is recognized that interactions between two weak bases are regulated by the free concentrations of the compounds in the cassette and the ability of these compounds to elevate intralysosomal pH (Daniel and Wojcikowski 1999b). Potential bioanalytical issues should be addressed when assembling the cassettes for investigation, so as to avoid technical hitches.

Preparation of Brain Slices and Incubation

It is important that the fresh brain slices are of high quality if the $V_{u,brain}$ values are to be relevant to the in vivo situation. This can be accomplished by keeping strictly to the protocol for preparation and maintenance of the brain slices during the experiment (Friden et al. 2009a; Loryan et al. 2013). The key steps of the brain slice method are illustrated in Fig. 13.2.

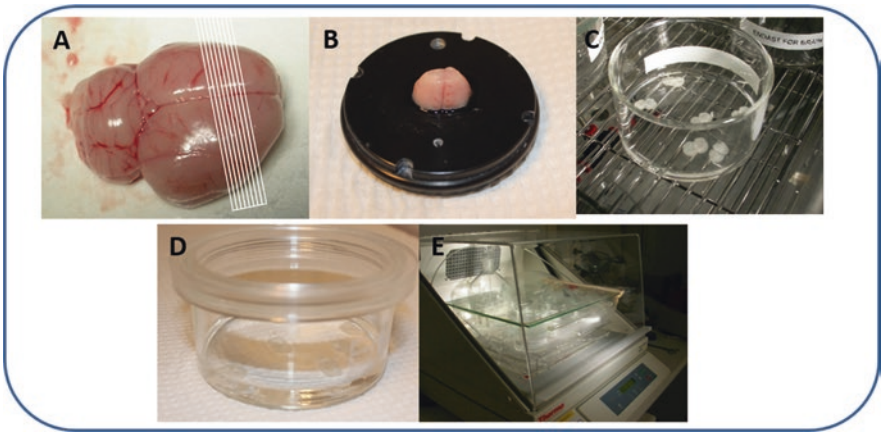

Fig. 13.2 An illustration of the main steps in the preparation of brain slices. (**a**) Schematic representation of the cutting direction. (**b**) The brain glued to the slicing platform in a coronal position. (**c**) Brain slices transferred into the Ø80-mm flat-bottomed glass beaker. (**d**) A beaker covered by the custom-fabricated lid fitted with a Teflon-fluorinated ethylene-propylene film. (**e**) The setup for the incubation-equilibration period. (Reprinted with permission from BioMed Central (Loryan et al. 2013))

The protocol for the fresh brain slice method (also called in vitro brain slice uptake technique) has not been unified among research laboratories, which makes comparison and interpretation of the results challenging. For instance, the brain can be sliced using a brain microslicer (Ooie et al. 1997; Benkwitz et al. 2007; Kodaira et al. 2011), a McIlwain tissue chopper (Becker and Liu 2006), or a vibratome (Friden et al. 2009a; Loryan et al. 2013). Moreover, researchers have used slices from different planes of the brain, such as the hypothalamic (Kakee et al. 1997), cortical (Kodaira et al. 2011) or striatal (Friden et al. 2009a, b). The thickness of the brain slices also differs between protocols: 300 μm (Kakee et al. 1996, 1997; Friden et al. 2009a, b), 400 μm (Becker and Liu 2006), or even 1000 μm (Van Peer et al. 1981). Accordingly, the incubation time (time required to reach equilibrium) varies and could be 8 h or longer, which may be too long to sustain the viability of the slices.

The time needed to reach equilibrium is influenced by various factors such as the amount of brain tissue per unit of the buffer volume, the stirring speed, and the initial concentration of the compound (Gredell et al. 2004; Benkwitz et al. 2007; Friden et al. 2009a). The ratio of six/ten (rat/mouse) 300 μm sequential brain slices to 15/10 mL (rat/mouse) of aECF has been found to be the most optimal for various diverse compounds to reach equilibrium in about 5 h (Friden et al. 2009a; Loryan et al. 2013). Very lipophilic compounds may require a longer equilibration time in some experimental setups, and this could compromise the viability of the brain slices. In this case, mathematical modeling of the data could be a reasonable alternative (Kodaira et al. 2011).

Sufficient viability of the brain slices is a critical prerequisite. The viability can be assessed indirectly by measuring the pH of the aECF (acidification of the medium is linked to low viability of the slices). However, more advanced methods such as measuring the ATP content of the slices (Friden et al. 2007; Kodaira et al. 2011; Uchida et al. 2011a) or the activity of released lactate dehydrogenase (Dos-Anjos et al. 2008; Loryan et al. 2013) are now recommended.

Bioanalysis

The drug concentrations in brain slices and aECF samples taken at equilibrium can be analyzed after homogenization using high-throughput techniques and LC-MS/MS as discussed for brain homogenate samples in Sect. 13.2.1.2.4. To avoid the preparation of calibration curves, 10- and 100-fold dilutions of the samples are preferable (Friden et al. 2009a). Several groups normalize the protein concentrations to correct for the dilution of brain homogenate (Kodaira et al. 2011).

In summary, the fresh brain slice method is a precise and robust technique for estimating the overall uptake of drugs into the brain tissue. This method is recommended for the estimation of target-site PK and toxicokinetics in the early drug discovery process in order to guide candidate selection (Friden et al. 2014; Loryan et al. 2014, 2017). One of the attractive features of the brain slice method is that it can be developed to investigate compound-specific molecular mechanisms of the intracerebral distribution of compounds (Friden et al. 2011; Chen et al. 2014; Puris

et al. 2019). For instance, fresh brain slices could be prepared from different strains of wild-type or genetically modified mice and rats to elucidate the effects of intracerebral transporters on the distribution of drugs within the brain (BBB transporters cannot be directly mapped with this technique). Furthermore, the brain slices could be manipulated genetically using various methods such as viral infection (Stokes et al. 2003) or biolistics (Wellmann et al. 1999). Disease models could also be used to study the diffusion and distribution of drugs or radiotracers within the brain (Newman et al. 1988b; Patlak et al. 1998). In addition, pharmacological inhibition or stimulation could be used to investigate particular distributional mechanisms, e.g., monensin or nigericin, to study the impact of lysosomal accumulation on the intracellular distribution of drugs (Friden et al. 2011; Logan et al. 2012).

13.4 Intracellular Distribution

Historically, it has been presumed that the transport of small molecules between intra- and extracellular neurocompartments is more efficient than BBB transport, which is considered to be a rate-limiting step for drug distribution to the brain (Wang and Welty 1996). Accordingly, from a PK point of view, the assessment of the intracerebral distribution of NCEs is usually less prioritized, is often inadequate because of a lack of reliable methods, and is narrowed to estimation of the unbound drug fraction in a brain homogenate, with subsequent evaluation of its half-life in the brain tissue (Liu et al. 2005). However, awareness of compound-specific intracerebral distributional mechanisms in early drug discovery could allow better directed evaluation and selection of drug candidates, based on the location of the potential CNS target (i.e., extra- or intracellular) and the probable side effects.

After passing the BBB, drugs are distributed in the extracellular space mainly by diffusion and convection (see Chap. 5 for a comprehensive analysis of the transport processes of drugs within the CNS). As pointed out in the state-of-the-art review by Wolak and Thorne (2013), the diffusion of molecules is governed by the features of the extracellular space (i.e., width, volume fraction, viscosity, geometry) as well as by any potential binding to the extracellular matrix or cellular membrane components (Fenstermacher and Kaye 1988). It should be highlighted that the diffusion of compounds in the extracellular neurocompartment is a potentially limiting step for macromolecules, nanoparticles, and viral vectors (Thorne et al. 2004, 2008; Thorne and Nicholson 2006). The bulk flow of the ISF should be accounted for in addition to the diffusion and hydraulic permeability (see Chap. 1 and Table 13.1). However, although it can be influential for poorly penetrating compounds, it is not a matter of concern for small highly lipophilic compounds (Cserr 1992; Davson 1995; Abbott 2004; Abbott et al. 2018). The bulk flow of the ISF has been measured as ~0.1–0.3 μL min^{-1} g^{-1} in the rat brain, but the actual value may be greater than this (Chap. 1 and Joan Abbott personal communication).

Because ISF is virtually protein free (Davson et al. 1970; Davson 1995), the drug present in the ISF can be measured as unbound and accessible for interactions at a

Table 13.1 Key components affecting drug distribution to and from the different compartments in the brain[a]

Extracellular neurocompartment	Cellular membranes	Intracellular neurocompartment
Diffusion in extracellular space Hydraulic permeability ISF bulk flow	Membrane permeation Active influx (e.g., organic cation transporters, L-type amino acid transporters) Active efflux Nonspecific binding to cell membrane components (often quantitatively insignificant) Specific binding to the target (often quantitatively insignificant)	Nonspecific binding to intracellular membrane components (often quantitatively significant) pH differences causing accumulation of weak bases in acidic compartments (e.g., lysosomes, endosomes) Specific binding to the target (e.g., tubulin, enzymes) Drug metabolism (often insignificant)[b]

[a]Differences between the types of brain parenchymal cells and brain subregions are not taken into account

[b]Drug metabolism is discussed in more detail in Chap. 6

cellular membrane level (Hammarlund-Udenaes et al. 1997; Ooie et al. 1997; Kalvass and Maurer 2002; Mano et al. 2002; Shen et al. 2004; Doran et al. 2005; Liu et al. 2005; Summerfield et al. 2006; Friden et al. 2007; Watson et al. 2009). The permeation of unbound, unionized drug through the cell membrane could be defined as the most significant distributional process of small molecules into the cell. Accumulation is a distributional process that is associated with asymmetry at the cellular barrier, is linked to the physiological pH gradient, and is driven by acidic intracellular compartments such as lysosomes, endosomes, peroxisomes, and the trans-Golgi network (Sect. 13.4.2). Asymmetry at the cellular barrier level can also occur as a consequence of active transport such as influx processes governed by organic cation transporters (e.g., 1-methyl-4-phenylpyridinium, tetraethylammonium, metformin) and L-type amino acid transporters (e.g., gabapentin), or efflux processes (Lee et al. 2001a, b; Bendayan et al. 2002; Kusuhara and Sugiyama 2002; Ohtsuki et al. 2004; Syvanen et al. 2012). The specific and nonspecific binding of compounds to extracellular constituents of the cell membrane can be ignored because of their much smaller surface areas, i.e., the external surface area of a typical human cell membrane represents less than 0.5% of the total cell membrane surface area (Freitas 1999).

After passing the cellular barrier, compounds can bind reversibly to intracellular constituents such as lipoproteins, phospholipids of the inner cellular membrane, or organelles. Nonspecific binding is often the dominant distributional component for small lipophilic compounds. In most cases, specific intracellular binding is irrelevant from a distributional perspective because of the low expression levels of the targets in relation to the extent of nonspecific binding. However, there are some exceptions; these are discussed at the end of Sect. 13.4.

Off-target or nonspecific binding of the drug to the cellular membranes is often not associated with any pharmacological response. However, progress has been made in recent decades toward an understanding of the interactions between the

ligand and the target (i.e., receptor, ion channel, enzyme). Primarily, the "passive" role of the cell membrane in target-binding kinetics has been questioned (Vauquelin and Packeu 2009). Novel membrane-connected concepts that reexamine the notion of the so-called nonspecific plasma membrane partitioning are being proposed (Sargent et al. 1988; Vauquelin and Van Liefde 2005). It has been recognized that nonspecific ligand-membrane interactions could be favorable, although not in all cases, for ligand-target interactions (Sargent and Schwyzer 1986; Bean et al. 1988; Vauquelin et al. 2012). This process could be very important for peptide-target interactions (Sargent and Schwyzer 1986). Another crucial aspect of membrane partitioning is the increased in vivo residence time of hydrophobic ligands. Slow release from the cell membranes is commonly acknowledged to be strongly associated with the long-lasting effects of highly lipophilic compounds (e.g., salmeterol). In other words, the cell membrane can be perceived as a depot/reservoir for hydrophobic ligands.

13.4.1 Using $K_{p,uu,cell}$ to Estimate the Extent of Cellular Barrier Transport

Frequently, as with plasma protein binding, scientists define the binding of drugs to brain tissue as "nonspecific." However, in comparison with plasma protein binding, less is known about the drug-brain tissue interaction, mainly because of technical difficulties in obtaining data on the tissue-binding components and in the quantification of intracellular drug concentrations.

In most cases, intracerebral distribution is assessed by either the ED-based brain homogenate method, with evaluation of $f_{u,brain}$, or the fresh brain slice method, with assessment of $V_{u,brain}$. Combining the two methods provides further information on intracellular distribution. The main determinant of $f_{u,brain}$ is *brain tissue binding* which primarily consists of nonspecific binding of the drug to various *intracellular* lipids and proteins. $V_{u,brain}$ then provides complementary data on intracerebral distribution factors other than binding. The importance of $K_{p,uu,cell}$ in this respect has been discussed by Friden et al. (2007, 2009a, 2011). $K_{p,uu,cell}$ can be estimated by combining $f_{u,brain}$ (brain homogenate) with $V_{u,brain}$ (brain slice) using Eq. 13.10 (Friden et al. 2007):

$$K_{p,uu,cell} = V_{u,brain} \cdot f_{u,brain} \tag{13.10}$$

$K_{p,uu,cell}$ describes the *steady-state* relationship of intracellular-to-extracellular unbound drug concentrations and provides the average concentration ratio for all cell types within the brain. The assumptions behind the $K_{p,uu,cell}$ concept are the following (Friden et al. 2007):

1. The ISF concentration is assumed to describe unbound drug (ISF is a practically protein-free fluid).

2. $C_{u,brainISF}$ represents the concentration of unbound drug in brain ISF from the entire brain (cranioregional and cell-type dissimilarities are not accounted for).
3. Membrane passive permeation and binding to intra- and extracellular constituents are the key distributional processes.
4. Intracellular drug molecules can be unbound or bound to intracellular components.
5. Drug binding to the outer part (surface) of the cell is negligible. However, this assumption could be incorrect for molecules with distribution entirely restricted to the ISF (e.g., large molecules) and/or those that are significantly bound to cellular membranes.

The derivation of the equations presented below is based on the definition of $V_{u,brain}$ as the ratio of the total amount of drug in the brain excluding the blood (A_{brain}) to the concentration of unbound drug in brain ISF (Eq. 13.8). According to the proposed distributional model (Friden et al. 2007), the total amount of drug in the brain can be presented as

$$A_{brain} = V_{brainISF} \cdot C_{u,brainISF} + V_{cell} \cdot V_{u,cell} \cdot C_{u,cell} \tag{13.11}$$

where $V_{brainISF}$ and V_{cell} are the physiological fractional volumes of ISF (~0.2 mL · g brain^{-1}) (Nicholson and Sykova 1998; Sykova and Nicholson 2008) and brain parenchymal cells (~0.8 mL · g brain^{-1}) and the density of brain tissue is assumed to be 1. $V_{u,cell}$ describes the volume of distribution of unbound drug in the cell (mL ICF · mL cell^{-1}) and relates the total amount of drug in the cell to the intracellular concentration of unbound drug; $C_{u,cell}$. $V_{u,cell}$ can be compared with $V_{u,brain}$, describing the whole brain drug distribution.

Another way of explaining $V_{u,cell}$ is that it describes the affinity of the drug to bind inside the cell. The more drug is bound, the higher the value of $V_{u,cell}$. It can be estimated using the ED-based brain homogenate method:

$$V_{u,cell} = 1 + \frac{D}{V_{cell}} \cdot \left(\frac{1}{f_{u,hD}} - 1 \right) \tag{13.12}$$

where $f_{u,hD}$ is the buffer-to-brain homogenate concentration ratio measured using ED and D is the dilution factor associated with homogenate preparation (Sect. 13.2).

Rewriting Eq. 13.8 using Eq. 13.11 and dividing both sides by $C_{u,brainISF}$ give

$$V_{u,brain} = V_{brainISF} + V_{cell} \cdot V_{u,cell} \cdot \frac{C_{u,cell}}{C_{u,brainISF}} \tag{13.13}$$

Consequently, the ratio of brain ICF to ISF unbound drug concentrations ($K_{p,uu,cell}$) can be derived as

$$K_{p,uu,cell} = \frac{C_{u,cell}}{C_{u,brainISF}} = \frac{V_{u,brain} - V_{brainISF}}{V_{cell} \cdot V_{u,cell}} \tag{13.14}$$

When analyzing numerical values of $K_{p,uu,cell}$, it is important to remember its meaning. When cellular membrane permeation is predominantly passive, the unbound drug intra- and extracellular concentrations are the same, giving a $K_{p,uu,cell}$ equal to unity. $K_{p,uu,cell}$ values higher than unity indicate intracellular accumulation, and $K_{p,uu,cell}$ values below unity could indicate active efflux at the cellular barrier. The estimation of $K_{p,uu,cell}$ is valuable for interpreting and understanding the processes governing the distribution of drugs into the brain parenchymal cells. It should be remembered, however, that the numbers obtained are average values from all the cell types in the brain.

13.4.2 Lysosomal Trapping

Although they were discovered in the early 1970s, the role of lysosomes in drug tissue distribution kinetics can still be considered as *terra incognita* (De Duve 1971). Lysosomes are conventionally acknowledged as the cell's "garbage disposal units." They are membrane-bound organelles containing about 50 hydrolytic enzymes that function at pH 4.5. Vacuolar-type H^+-ATPase embedded in the lysosomal membrane maintains the intralysosomal acidic environment.

Lysosomotropism or *lysosomal trapping* is a phenomenon where compounds (*lysosomotropic agents*) with both a lipophilic moiety and a basic moiety are accumulated in acidic intracellular compartments mainly in lysosomes (Fig. 13.3) (De Duve 1970; Nadanaciva et al. 2011).

Lysosomal trapping is governed by the large physiological pH gradient between ICF and lysosomes. The process of lysosomal trapping is saturable, energy-dependent (necessary for the normal function of the H^+-ATPase), and requires cellular integrity (De Duve 1970; MacIntyre and Cutler 1988; Daniel and Wojcikowski 1999a). Weak bases in their unionized state permeate cellular and lysosomal membranes and accumulate in the acidic compartment of lysosomes (Fig. 13.4). Diacidic bases are trapped more easily than monoacidic bases, with a subsequent impact on their distribution (MacIntyre and Cutler 1988). Because they are protonated within the lysosomes, the bases are not able to diffuse back into the cytosol (MacIntyre and Cutler 1988; Lloyd 2000; Kaufmann and Krise 2007). The intralysosomal concentrations of trapped compounds can reach high levels, with lysosome-to-cytosol accumulation ratios as high as 100:1 (Daniel and Wojcikowski 1997). Moreover, because the weak bases interact with phospholipids within the lysosome, the apparent lysosomal volume measured indirectly could be substantially greater than the physical (i.e., actual) lysosomal volume (MacIntyre and Cutler 1988; Duvvuri and Krise 2005). The physical volume of the lysosomes can also increase with time due

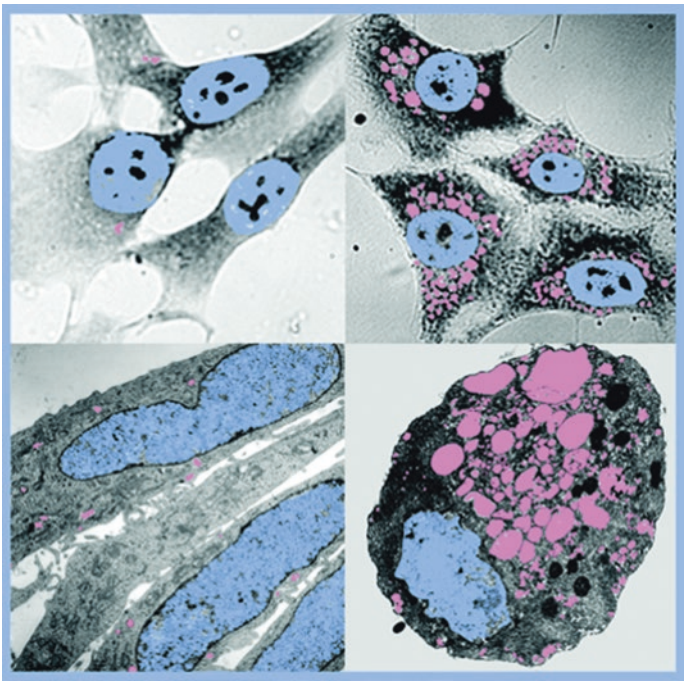

Fig. 13.3 Cells displaying the lysosomal trapping phenomenon. Picture from Boya et al. (2003). In contrast to controls (top left and bottom left panels), cells treated with the lysosomotropic drug ciprofloxacin (top right and bottom right panels) manifest multiple autophagic vacuoles (colored pink) in the cytoplasm, before undergoing apoptosis. The bottom microphotographs have been obtained by electron microscopy, while the top ones result from conventional light microscopy, after Giemsa staining. Nuclei are colored blue. (Reprinted with permission from Rockefeller University Press (picture appeared on the cover page of J Exp Med, May 19, 2003))

to vesicle-mediated trafficking and fusion of lysosomes with the cell membrane (Kaufmann and Krise 2007; Logan et al. 2012).

Consequently, despite the very small physiological volume of the lysosomes (~ 0.01 mL · g brain^{-1}), lysosomotropic compounds show extensive tissue accumulation (e.g., in the lungs, liver, and brain) which is reflected by a high apparent volume of distribution (Daniel and Wojcikowski 1999b). Moreover, lysosomal trapping can result in drug-drug interactions (Daniel et al. 1995, 1998, 2000; Daniel and Wojcikowski 1999b; Logan et al. 2012). For instance, because the process of lysosomal trapping is saturable, the lysosomal uptake of co-administered drugs could decline. All this suggests that lysosomal trapping is an important mechanism of drug distribution with potential impact on systemic PK.

Although the brain tissue is not as lysosome-rich as the lungs, liver, and kidneys, lysosomal trapping could also influence the brain PK. Many marketed and novel neurotherapeutics are cationic amphiphilic compounds; it is thus not surprising that they are lysosomotropic (Daniel 2003; Nadanaciva et al. 2011). Hence, it is

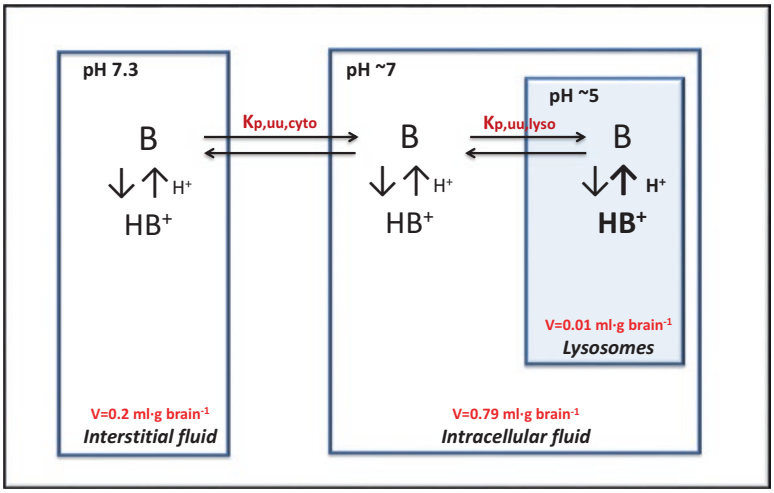

Fig. 13.4 Graphic illustration of the pH partitioning of a basic drug between extra- and intracellular compartments, i.e., interstitial fluid, intracellular fluid, and lysosomes. Accumulation of the protonated form (HB+) of the basic drug (B) in the compartments is driven by the physiological pH gradient. The cytosolic-to-interstitial fluid unbound drug concentration ratio ($K_{p,uu,cyto}$) and the lysosomic-to-cytosolic unbound drug concentration ratio ($K_{p,uu,lyso}$) can be estimated using a three-compartment pH partitioning model (Friden et al. 2011)

recommended that particular attention be paid to lysosomotropism in CNS drug development programs.

Lysosomotropism is also interesting in that there are several lysosomal acidic hydrolases that may be useful pharmacological CNS targets (de Duve 1975; Boya and Kroemer 2008; Schultz et al. 2011). For instance, acid sphingomyelinase affects ceramide levels in several psychiatric and neurological disorders such as major depression, morphine antinociceptive tolerance, and Alzheimer's disease (Schwarz et al. 2008; Ndengele et al. 2009; He et al. 2010; Kornhuber et al. 2011). The inhibition of acid sphingomyelinase results in anti-apoptotic, proliferative, and anti-inflammatory effects. Consequently, functional acid sphingomyelinase inhibitors have potential in a number of new clinical therapies (Muehlbacher et al. 2012).

13.4.2.1 Compensation for pH Partitioning

Several researchers have suggested that lysosomal accumulation is a potential explanation for dissimilarities between in vitro (homogenates) and in vivo measurements when describing the distribution of acidic, neutral, and basic drugs in tissues other than the brain (Harashima et al. 1984; Sawada et al. 1984; MacIntyre and Cutler 1988; Daniel and Wojcikowski 1997; Yokogawa et al. 2002; Maurer et al. 2005). For instance, it has been documented that predictions of the pharmacokinetic parameter apparent volume of distribution at steady state (V_{ss}) for 36 compounds,

based on measurement of the unbound drug fraction in 15 different tissues, were less accurate for acidic and strongly basic substances (Berry et al. 2010). However, after making allowance for the ionic effects of tissue-to-blood pH gradients, the predictions for V_{ss} were accurate within a threefold range for 81% of the compounds studied.

Inconsistencies between $C_{u,brainISF}$ values obtained using cerebral microdialysis and those projected from A_{brain} corrected for nonspecific binding using $f_{u,brain}$ for weak bases and acids are also thought to be linked to lysosomotropism (Friden et al. 2007). Lysosomotropism in the brain tissue is also important when comparing brain slice and brain homogenate data (Friden et al. 2011; Loryan et al. 2015, 2017).

The cell partitioning coefficient frequently deviates from unity. Intracellular accumulation as a result of the pH gradient is often suggested as one of the main reasons for the mismatch between the brain homogenate and brain slice data, i.e., $f_{u,brain} \neq 1/V_{u,brain}$. The lack of agreement is mainly due to the different properties of the two methods; cell and organelle membranes are retained in the slices, and pH differences are preserved. If the intracellular unbound drug concentration is similar to the brain ISF unbound drug concentration (i.e., $K_{p,uu,cell}$ is close to unity and $V_{u,brain}$ exceeding 1 mL · g brain^{-1}), it can be assumed that intracellular nonspecific binding to membrane constituents is a major, quantitatively significant, distributional mechanism.

If only $K_{p,uu,brain}$ is of interest and brain homogenate data are used, the $f_{u,brain}$ values can be corrected to more in vivo-like values by compensating for pH partitioning according to the pKa of the drug (Friden et al. 2007, 2011; Loryan et al. 2014).

A three-compartment (ISF, cytosol, and lysosomes) pH partitioning model for $K_{p,uu,cell}$ based on the strong relationship between drug accumulation in acidic compartments due to lysosomal trapping and the pKa values of the compound has been developed (Fig. 13.4) (Friden et al. 2011; Loryan et al. 2015). The starting point is described by Eq. 13.15:

$$K_{p,uu,cell} = V_{u,brain} \cdot f_{u,brain} = \frac{A_{brain}}{C_{u,brainISF}} \cdot f_{u,brain} \tag{13.15}$$

The total amount of drug in the brain can be described as the sum of the total amounts in the ISF, the cytosol, and the lysosomes, denoted as A_{ISF}, A_{cyto}, and A_{lyso}, respectively. Each compartment is described by its physiological volume multiplied by the concentration of unbound drug in the compartment, divided by $f_{u,brain}$:

$$A_{brain} = A_{ISF} + A_{cyto} + A_{lyso} = \frac{V_{brainISF} \cdot C_{u,brainISF} + V_{cyto} \cdot C_{u,cyto} + V_{lyso} \cdot C_{u,lyso}}{f_{u,brain}} \tag{13.16}$$

V_{ISF}, V_{cyto}, and V_{lyso} are the physiological volumes of the ISF (0.20 mL · g brain^{-1}), cytosol (0.79 mL · g brain^{-1}), and lysosomes (0.01 mL · g brain^{-1}), respectively. $C_{u,cyto}$ and $C_{u,lyso}$ describe the unbound drug concentrations in cytoplasm and lysosomes, respectively. If Eqs. 13.15 and 13.16 are combined, $K_{p,uu,cell}$, predicted from the three-compartment pH partition model, can be defined as

$$K_{p,uu,cell} = V_{ISF} + K_{p,uu,cyto} \cdot \left(V_{cyto} + V_{lyso} \cdot K_{p,uu,lyso}\right) \tag{13.17}$$

The estimation of the cytosolic-to-interstitial fluid unbound drug concentration ratio ($K_{p,uu,cyto}$) and the lysosomic-to-cytosolic unbound drug concentration ratio ($K_{p,uu,lyso}$) can be computed by introducing the pKa values of the compounds (i.e., bases) in Eqs. 13.18 and 13.19, respectively:

$$K_{p,uu,cyto} = \frac{C_{u,cyto}}{C_{u,brainISF}} = \frac{10^{pKa-pH_{cyto}} + 1}{10^{pKa-pH_{ISF}} + 1} \tag{13.18}$$

$$K_{p,uu,lyso} = \frac{C_{u,lyso}}{C_{u,brainISF}} = \frac{10^{pKa-pH_{lyso}} + 1}{10^{pKa-pH_{cyto}} + 1} \tag{13.19}$$

where pH_{cyto} = 7.06, and pH_{lyso} = 5.18, as determined by Friden and co-workers (Friden et al. 2011).

The main application of the pH partitioning model is related to $f_{u,brain}$, measured using the brain homogenate method. Based on pH partitioning, $V_{u,brain}$ ($1/f_{u,brain,corrected}$) can be estimated from $f_{u,brain}$ using Eq. 13.15, i.e., by dividing the calculated $K_{p,uu,cell}$ by $f_{u,brain}$ (Eq. 13.17). As demonstrated after the correction for pH partitioning, the discrepancy between brain homogenate and brain slice methods was practically abolished in a dataset consisting of 56 compounds (Friden et al. 2011). However, the pH partitioning model was still incapable of identifying and/or correcting other processes governing the dissimilarities between the brain slice and homogenate methods, such as active uptake into the cells.

The three-compartment pH partitioning model can also be used for the preliminary evaluation of $K_{p,uu,cell}$ and identification of potential lysosomotropic compounds already in the lead optimization phase (Friden et al. 2011; Loryan et al. 2014, 2017). pKa values are frequently calculated in silico in the early discovery stages, and a critical approach is recommended since they may not reflect the real pKa values. pKa values measured at 25 °C can also diverge from actual in vivo values when using the pH partitioning model (Sun and Avdeef 2011). This can lead to some differences in experimental $K_{p,uu,cell}$ and computed $K_{p,uu,cell}$ values.

13.4.3 Intracerebral Distributional Patterns

Because of the physicochemical features and character of the pharmacological targets, the patterns of intracerebral distribution can differ for different drugs (Fig. 13.5). Thioridazine, salicylic acid, and gabapentin are used as model drugs and are discussed in detail in this section.

Figure 13.5a shows the intracerebral distribution of thioridazine. Thioridazine is a base, with a pKa of 8.9 and pronounced plasma and brain tissue binding mainly as

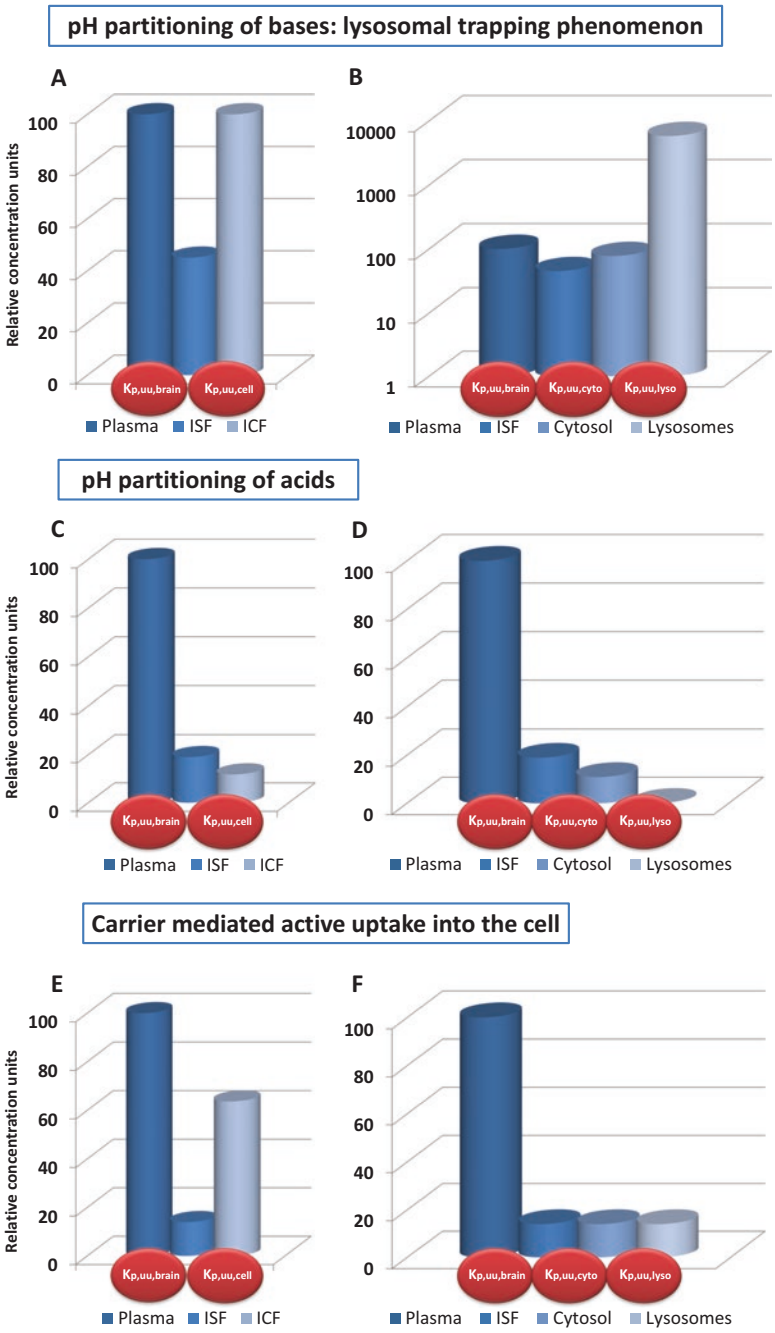

Fig. 13.5 The intracerebral unbound drug distribution patterns of prototypical drugs (**a** and **b**, thioridazine; **c** and **d**, salicylic acid; **e** and **f**, gabapentin). The distributional pattern depends on both the physicochemical properties of the compound and the functional characteristics of the

a result of its high lipophilicity (ClogP 6.0). The experimental $f_{u,plasma,rat}$ is 0.002, and the $V_{u,brain}$ is around 3000 mL · g brain^{-1}, the highest $V_{u,brain}$ observed so far (Friden et al. 2009a; Loryan et al. 2013). As a result, the determined $K_{p,brain}$ of 3.75 is significantly influenced by nonspecific binding to the brain tissue and plasma proteins. The $K_{p,uu,brain}$ is 0.45 (Friden et al. 2009b). *Lysosomal trapping* is the main reason for thioridazine accumulating in the cells. When the intracellular compartment is viewed as one unit, there is a 2.24-fold higher intracellular concentration of unbound thioridazine than in the brain ISF. Moreover, because of the presence of the physiological pH gradient, thioridazine as a base accumulates in the cytosol and then becomes trapped in the acidic intracellular compartments (Fig. 13.5b). The calculations (Eq. 13.19) indicate that, when the cytosolic compartment is separated from the lysosomal compartment, thioridazine will reach a 75-fold higher intralysosomal than cytosolic concentration. This type of distribution could be considered as a *signature pattern* for basic compounds.

Acidic compounds such as salicylic acid (see Fig. 13.5c) have a different distribution pattern in the brain. Only 19% of the unbound salicylic acid in the plasma crosses the BBB ($K_{p,uu,brain}$ = 0.19). Moreover, about 60% of the unbound salicylic acid in brain ISF equilibrates across the cellular barrier. Using the three-compartment pH partitioning model (Fig. 13.5d), it is possible to describe the unbound cytosolic and lysosomal partitioning coefficients and identify a lysosomal exclusion phenomenon ($K_{p,uu,lyso}$ = 0.015).

Active carrier-mediated transport into the cells is an alternative process which can be observed at the cellular barrier. Gabapentin provides a classic example of a compound lacking any nonspecific binding to the brain tissue ($f_{u,brain}$ = 1) while at the same time exhibiting active uptake into the cells (Fig. 13.5e). Due to the active passage of gabapentin into the cells by the L-α-amino acid transporter (Su et al. 1995), it reaches nearly five-fold higher intracellular concentrations on average. Additional examples of compounds undergoing active cellular uptake include

Fig. 13.5 (continued) compartments and membranes, defined by $K_{p,uu,brain}$ and $K_{p,uu,cell}$. The graphs were constructed from data in Friden et al. (2007, 2011). The unbound drug plasma concentration is set at 100 arbitrary units

(**a**) Efflux of thioridazine at the BBB ($K_{p,uu,brain}$ = 0.45) and its accumulation in the cells as described by a $K_{p,uu,cell}$ of 2.24. Because thioridazine is a weak base with a pKa of 8.9, it is subject to lysosomal trapping and accumulation in the cells. The pH partitioning of thioridazine (**b**) is described by the unbound thioridazine cytosolic ($K_{p,uu,cyto}$=1.72) and lysosomal ($K_{p,uu,lyso}$=75) partition coefficients computed using the three-compartment pH partitioning model. (**c** and **d**) The distribution and pH partitioning of salicylic acid. Salicylic acid is poorly transported across the BBB ($K_{p,uu,brain}$ = 0.19) and has reduced cellular penetration ($K_{p,uu,cell}$ = 0.62). Moreover, as an acid (pKa 4.3), salicylic acid has limited distribution in the brain tissue ($V_{u,brain}$ = 1 mL · g brain^{-1}). The pH partitioning model (**d**) supports the suggestion that salicylic acid is mainly distributed in the cytosol ($K_{p,uu,cyto}$ = 0.58) and is almost completely absent from acidic compartments such as lysosomes ($K_{p,uu,lyso}$ = 0.015). (**e** and **f**) The zwitterion gabapentin. Gabapentin transport in the BBB is restricted ($K_{p,uu,brain}$ = 0.14). However, after passing the BBB, it is excessively accumulated in the cells ($K_{p,uu,cell}$ = 4.55). The pH partitioning model is, however, incapable of identifying its uptake in the cells since the uptake is not related to lysosomal accumulation. Gabapentin is a substrate of the L-type amino acid transporter, which explains the observed active uptake into the cells. (Wang and Welty 1996; Friden et al. 2011)

1-methyl-4-phenylpyridinium (MPP, $K_{p,uu,cell}$ = 77) and tetraethylammonium (TEA, $K_{p,uu,cell}$ = 8.95) (Friden et al. 2011).

Because of the practical value of $K_{p,uu,cell}$ and its further division into $K_{p,uu,lyso}$ and $K_{p,uu,cyto}$, it is highly recommended that the unbound drug intra-to-extracellular concentration ratio be assessed in DMPK studies. Estimated neuroPK parameters are important contributors to the evaluation of the intracerebral distribution pattern of NCEs and their possible side effects.

13.5 Combinatory Mapping Approach

$K_{p,brain}$ estimated under steady-state conditions or using the area under the concentration-time curves in the brain tissue ($AUC_{tot,brain}$) and plasma ($AUC_{tot,plasma}$) after a single dose (Eq. 13.20) has historically been recognized as a driving force in CNS drug discovery screening programs (Pardridge 1989; Ghose et al. 1999, 2012) (see Chap. 7 for an explanation of the doctrines of brain PK).

$$K_{p,brain} = \frac{A_{brain}}{C_{tot,plasma}} = \frac{AUC_{tot,brain}}{AUC_{tot,plasma}} \tag{13.20}$$

The identification and selection of drug candidates with "acceptable brain penetration" has typically been based on pre-defined cut-off values for $K_{p,brain}$ (BB or often logBB); however, these vary between groups/companies. For instance, logBB = 0.3 ($K_{p,brain}$ = 2) has often been used as the cut-off point for NCE penetration of the BBB (Reichel 2006). Another approach uses an arbitrary cut-off point for $K_{p,brain}$ of greater than unity (Kalvass et al. 2007a; Padowski and Pollack 2011a). At Eli Lilly research laboratories, the cut-off point for $K_{p,brain}$, determined using a mouse brain uptake assay, was 0.3 (30%) (Raub et al. 2006). Alternatively, substances with $K_{p,brain}$ values higher than 0.04 (determined using the brain tissue with residual blood) have been considered "brain penetrants" by some, since this value exceeds the cerebral blood volume, approximated as 4% of the total brain volume (Hitchcock and Pennington 2006; Shaffer 2010). Basically, higher $K_{p,brain}$ values have frequently been considered to be favorable for CNS penetration (Young et al. 1988; Pardridge 1989; Ghose et al. 1999, 2012; Segall 2012). Despite the fact that it has been found to be inadequate for evaluation of the transport of drugs across the BBB and to be by no means foolproof, this type of "taxonomy" has been common practice in the pharmaceutical industry.

However, off-target binding of drug to plasma and brain tissues irrefutably masks the actual BBB net flux (see Chaps. 6, 13, and 14 for more detailed explanations). Currently, driven by abundant evidence supporting the "free-drug hypothesis," $K_{p,uu,brain}$ (also called $K_{p,free}$) is replacing $K_{p,brain}$.

Several scientists have tried to differentiate between the two main components of $K_{p,brain}$, i.e., nonspecific binding to tissues and free (unbound) drug (Lin et al. 1982; Kalvass and Maurer 2002; Mano et al. 2002; Maurer et al. 2005; Gupta et al. 2006;

Summerfield et al. 2006; Friden et al. 2007; Hammarlund-Udenaes et al. 2008; Liu et al. 2009a). For instance, Becker and Liu categorize the ratio of $f_{u,plasma}$ to $f_{u,brain}$ as an "intrinsic" partition coefficient between the brain and plasma ($K_{p,in}$) which could be considered a descriptor of nonspecific binding in brain and plasma (Becker and Liu 2006). It is, however, essential to bear in mind that $K_{p,in}$ and $K_{p,uu,brain}$ describe different properties of the compound, where $K_{p,in}$ describes the ratio of the binding properties without including BBB transport (if there is no observed active transport, $K_{p,brain} = K_{p,in}$), and $K_{p,uu,brain}$ specifically defines the BBB transport of unbound drug. $K_{p,in}$ cannot therefore be used to assess the $K_{p,uu,brain}$ of NCEs.

Alternative approaches to the use of microdialysis for determining $K_{p,uu,brain}$ that are based on the co-estimation of $K_{p,brain}$ and nonspecific binding to plasma and brain tissues have been established (Gupta et al. 2006; Friden et al. 2007; Hammarlund-Udenaes et al. 2008, 2009). Hence, $f_{u,plasma}$ can be used to correct $C_{tot,plasma}$ (binding to formal elements of the blood is excluded):

$$C_{u,plasma} = C_{tot,plasma} \cdot f_{u,plasma} \tag{13.21}$$

Correspondingly, $V_{u,brain}$ (mL · g brain^{-1}) or $f_{u,brain}$ corrected for pH partitioning ($f_{u,brain,corrected}$) is used to estimate $C_{u,brainISF}$ (μmol · g brain^{-1}):

$$C_{u,brainISF} = \frac{A_{brain}}{V_{u,brain}} = A_{brain} \cdot f_{u,brain,corected} \tag{13.22}$$

Accordingly, $K_{p,uu,brain}$ can be derived from Eq. 13.20 as

$$K_{p,uu,brain} = \frac{K_{p,brain}}{V_{u,brain} \cdot f_{u,plasma}} = \frac{K_{p,brain}}{\frac{1}{f_{u,brain,corrected}} \cdot f_{u,plasma}} \tag{13.23}$$

Because this method (Eq. 13.23) is based on several individually determined parameters obtained using various techniques, the level of uncertainty and variability in the final $K_{p,uu,brain}$ estimates is increased. Therefore, reduction of the potential uncertainty in each measurement ($K_{p,brain}$, $V_{u,brain}$, $f_{u,brain}$, $f_{u,plasma}$) will make assessment of the brain partitioning coefficient for unbound drug more secure in drug discovery. Some critical steps in determining the brain partitioning coefficient for total drug, required for the assessment of $K_{p,uu,brain}$, are described below.

Ideally, the brain partitioning coefficient would be determined using steady-state total brain and plasma concentrations after constant-rate intravenous infusion (Friden et al. 2009b; Hammarlund-Udenaes et al. 2009). However, in drug discovery and development setups, intravenous infusions can be challenging and consequently are often not an option. Alternatively, $K_{p,brain}$ can be determined as the $AUC_{tot,brain}/AUC_{tot,plasma}$ ratio (Eq. 13.20), using various time points (up to five animals per time point) after a single (discrete) dose. In fact, subcutaneous administration is most commonly used, because it decreases the inter-experimental variability,

mainly as a result of the compounds circumventing oral absorption and first-pass metabolism. In some cases, $K_{p,brain}$ is assessed using total brain and plasma concentrations obtained at a specific point in time after drug administration. However, this approach has been heavily criticized since it is known that $K_{p,brain}$ is a time-dependent parameter (Padowski and Pollack 2011a, b). In this regard, Padowski and Pollack have suggested the use of different notations of $K_{p,brain}$ with the intention of specifying the conditions under which brain exposure has been determined, i.e., $K_{p,brain,t}$ (single time point), $K_{p,brain,DE}$ (distributional equilibrium reached), and $K_{p,brain,SS}$ (in a steady state system) (Padowski and Pollack 2011a). These researchers have also used a simulation approach to study the links between $K_{p,brain,t}$ with a sampling time prior to the point of distribution equilibrium and the experimentally obtained $K_{p,brain}$ in the presence vs absence of P-gp efflux transport. In some cases, an initial overshoot or increase in $K_{p,brain,t}$ values was followed by a decline to a value which remained constant with time. Consequently, it was concluded that the P-gp effect estimated based on a $K_{p,brain}$ value prior to reaching distribution equilibrium could be significantly inaccurate. The experimental design will thus greatly influence the conclusions made. The simulations also indicated that assessment of the P-gp effect was more precise and less variable with intravenous constant-rate infusions than with bolus administration, i.e., that $K_{p,brainSS}$ was the most appropriate choice (Gibaldi 1969; Padowski and Pollack 2011b). Although the proposed ranking of these parameters certainly introduces clarity and flags the importance of potential time-dependent differences in BBB equilibration, it has not been followed up in practice to any great extent (in this chapter $K_{p,brain}$ refers to $K_{p,brain,ss}$).

The correlation between $K_{p,brain}$ derived from a single (discrete) dose and that derived at steady-state has also been investigated in an attempt to improve throughput in neuroPK studies in the industrial setting. For instance, $K_{p,brain}$ values derived from a single dose differed maximally 2.5-fold from the steady-state values for eight of the nine commercial and two proprietary compounds tested (>2.5-fold for thiopental) (Liu et al. 2009a; Doran et al. 2012). These results give the impression that the single-dose approach, which is more time-efficient, may not compromise data quality to any great extent.

Another approach, which was introduced with the intention of reducing the use of animals and improving the efficiency of investigations into CNS exposure in drug discovery programs, uses a mixture of up to five NCEs administered together, termed a *cocktail*, *cassette*, or *Nin1* (Manitpisitkul and White 2004; Friden et al. 2009b; Liu et al. 2012). Liu and colleagues investigated the brain partitioning coefficients of 11 model compounds using discrete and cassette dosing and discovered that drug-drug interactions at the BBB level are unlikely at these low subcutaneous cassette doses (Liu et al. 2012). Nevertheless, it is advisable to administer low doses of the drugs during the experiment to prevent any interactions at the BBB as well as potential side effects. Overall, the route and duration of administration, the dose (discrete or cassette dosing), and the brain and plasma tissue sampling times should be critically evaluated prior to the experiment to avoid potential pitfalls.

Methods for correcting the residual blood in the sampled brain tissue also need to be considered. Using $V_{u,brain}$ to determine $K_{p,uu,brain}$, Friden et al. showed that the literature values for V_{blood} may be too high when used for correcting A_{brain} for the residual blood (Friden et al. 2010). This was especially observed for drugs with low $K_{p,brain}$ values. A low $K_{p,brain}$ value can be caused by either very efficient efflux at the BBB or plasma protein binding that greatly exceeds the nonspecific binding of the drug in the brain. The latter becomes a problem when using a value for V_{blood} that is too high. An improved method has been developed for this estimation (Friden et al. 2010). It should be noted that the remaining brain vascular space varies with the method used to sacrifice the animal.

The correction for residual blood can be calculated from the effective plasma space in the brain for a given drug, V_{eff}, which in turn can be calculated from the measured plasma protein binding according to

$$V_{eff} = f_{u,plasma} \cdot V_{water} + \left(1 - f_{u,plasma}\right) \cdot V_{protein} \tag{13.24}$$

V_{water} and $V_{protein}$ in rat brain capillary blood have been estimated as 7.99 μl · g brain^{-1} and 10.3 μl · g brain^{-1}, respectively (Friden et al. 2010). This equation can be used when binding to blood elements is not significant. The amount of drug in the brain tissue excluding the capillary contents, A_{brain}, can be calculated as

$$A_{brain} = \frac{C_{tot,brain} - V_{eff} \cdot C_{tot,plasma}}{1 - V_{water}} \tag{13.25}$$

$C_{tot,brain}$ is the concentration of drug in the whole brain tissue sample, and $C_{tot,plasma}$ is the drug concentration in a regular (arterial) plasma sample. The total physical volume of residual blood in the rat brain after exsanguination by severing the heart has been estimated as 12.7 μl · g brain^{-1} (Friden et al. 2010).

The complexity of the processes governing the drug concentrations in the brain requires the input of several methods, each providing a defined piece of the information required to assemble a more in-depth picture of drug disposition in the CNS on the level of the entire brain or the brain regions of interest (Loryan et al. 2014, 2016). Using the CMA, it is possible in the early drug development phases to map the concentrations of unbound drug in the main pharmacokinetic compartments relevant to drug disposition in the brain, such as plasma, ISF, ICF (and if necessary lysosomes), and CSF. The compartments and relevant concentration relationships are illustrated in Fig. 13.6, using the atypical antidepressant bupropion as a model.

The main benefit of this mapping approach is the visualization and better understanding of the target site PK. Additionally, it allows the ranking of the compounds based on the target compartment unbound drug concentration normalized by the PD parameters (EC50, IC50, Ki, etc.) as well as in the design of new PK/PD studies.

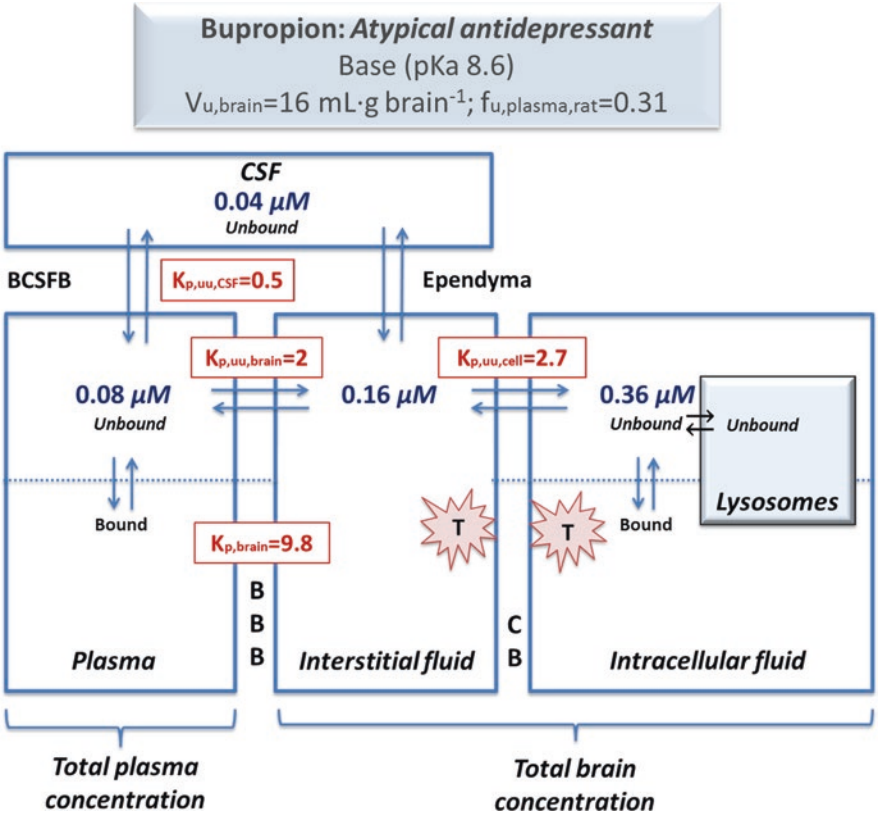

Fig. 13.6 Schematic representation of the distribution of a drug, here exemplified by the atypical antidepressant bupropion, into the different compartments (plasma, brain ISF, brain ICF, lysosomes, and CSF) involved in the disposition of drugs across the barriers (BBB, CB, and BCSFB), with the resulting concentrations obtained in each compartment. T represents the possible target sites of the drug, facing either the ISF or the ICF. The graph was constructed using steady-state total plasma, total brain, and CSF concentration determinations in rats after a 4-h constant-rate intravenous infusion of bupropion 2 (μmol/kg)/h (Friden et al. 2009b). Using this model and given the unbound drug plasma concentration, it is possible to estimate the target site concentrations. This approach can be used in drug discovery programs for establishing the link between the PK and engagement of the target. The $K_{p,uu,CSF}$ is quite different from the $K_{p,uu,brain}$ for bupropion, which means that estimations of the target site concentrations will be less valuable if based on CSF measurements

13.6 Translational Aspects of the Methods

In the drug discovery process, in vitro assays and preclinical animal studies are widely used to evaluate the potency of NCEs and to identify candidates that may have desirable clinical responses.

However, when there is no correlation between in vivo and in vitro potencies, the validity of the in vitro assay, the animal model, and the target can be questioned

(Brunner et al. 2012). Translational science is the study of the extrapolation of experimental findings to clinical solutions. It is important to improve the proficiency of clinical trial design by planning clinical doses based on nonclinical results. Animal brain PK studies are a routine tool for predicting drug behavior in humans. Thus far, it has been extremely challenging to master the translation of in vitro-to-in vivo and animal-to-human data in the drug discovery process, primarily because of the shortage of supportive data and the underlying multiple assumptions. Some translational aspects linked to methodologies described in this chapter are discussed below.

13.6.1 Translational Aspects of Brain Tissue Binding Assays

It is important to estimate the cerebral concentrations of unbound neurotherapeutic drugs in various species and related these to the potential CNS activity and target engagement of the drugs in preclinical and clinical PK studies. The $f_{u,brain}$ of drug candidates is routinely determined in several species to account for possible species dependence, as is the case with plasma protein binding, although this does not fit with experimental results demonstrating that brain tissue binding is less sensitive to interspecies dissimilarities than plasma protein binding (Summerfield et al. 2007; Wan et al. 2009; Read and Braggio 2010). In fact, when Di et al. evaluated the degree and nature of potential species differences in brain tissue binding, they found that brain tissue binding is species independent when studying healthy mammals (Di et al. 2011). This finding was very beneficial for translational medicine because it meant that a single representative species such as the rat could replace multispecies determinations of $f_{u,brain}$. However, a recent study on drug brain-regional brain tissue binding investigated in postmortem material obtained from patients with Alzheimer's disease revealed extensive intra- and interindividual variability that is more pronounced in disease conditions (Gustafsson et al. 2019). The findings highlight the need of investigation of brain tissue binding also in pathological conditions.

Laboratory studies have not found any significant dissimilarities in estimated $V_{u,brain}$ values from fresh brain slices between Sprague-Dawley rats and NMRI mice (Fig. 13.7). However, more systematic investigations are required to support the possibility of the interchangeable use of $V_{u,brain}$ measurements for translational studies.

13.6.2 Translational Aspects of Brain Exposure Assessment

In the drug industry, the translation of drug tissue distribution data between species is grounded on the assumption that the tissue-to-plasma drug partitioning coefficient for passive transport is tissue- and species-independent. However, the available information in the literature supports the existence of interspecies differences

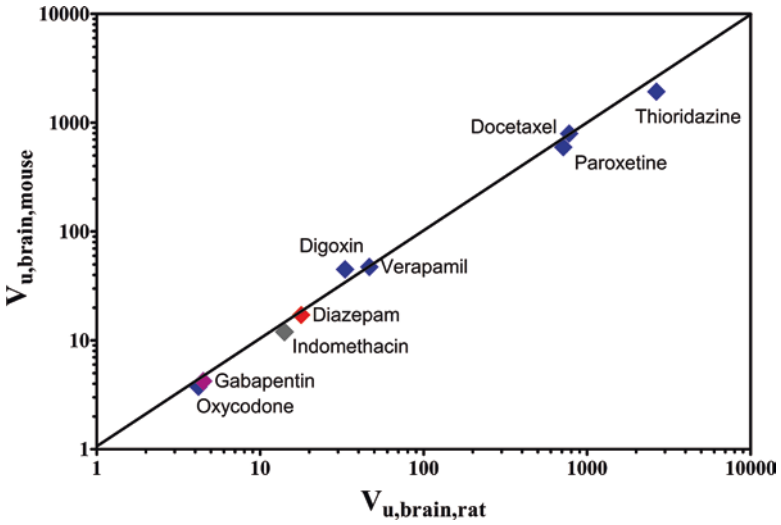

Fig. 13.7 The relationship between rat (x-axis) and mouse (y-axis) brain slices for the estimation of the volume of distribution of unbound drug in the brain of ten compounds ($V_{u,brain}$; mL · g brain^{-1}). The solid line represents the line of identity. The color of the diamonds represents the ion class of the compound (bases: thioridazine, docetaxel, paroxetine, verapamil, digoxin, oxycodone; neutral: diazepam; acid: indomethacin; zwitterion: gabapentin). Constructed from data in Loryan et al. (2013)

in the lipid composition of the tissues, which is considered to be the main factor in drug binding to tissues (Rouser et al. 1969; Simon and Rouser 1969). Elaborate investigation of tissue lipid composition with regard to drug distribution in dogs and rats has demonstrated clear differences between the animals; e.g., the proportion of neutral lipids was fivefold lower in dog brain than in rat brain (Rodgers et al. 2012). The authors suggested that the assumption of constancy in tissue-to-plasma partitioning should be used with caution when species-specific tissue distribution is of interest. Nevertheless, based on a widely accepted measure of prediction that describes the number of compounds that fall within a two- to threefold range, various groups have demonstrated the reliability of rodent-derived PK parameters for predicting BBB net flux in humans and large animals, although this has mainly been for compounds with predominantly passive transport (Friden et al. 2009b; Di et al. 2012a; Doran et al. 2012; Kielbasa and Stratford Jr. 2012; Westerhout et al. 2012). For instance, Doran and colleagues showed that preclinical rat-derived neuroPK parameters, particularly $K_{p,uu,brain}$, can be used to extrapolate $C_{u,brainISF}$ in dogs and nonhuman primates for freely permeating non-P-gp substrates (Doran et al. 2012). In contrast, the prediction of $C_{u,brainISF}$ for P-gp substrates such as risperidone and 9-hydroxyrisperidone using a similar approach was significantly flawed, with underprediction of $K_{p,uu,brain}$ in dogs and nonhuman primates from rat-derived data. Several reports describing species differences in brain exposure measurements have also been documented (Dagenais et al. 2001; Syvanen et al. 2008; Syvanen et al.

2009; Bundgaard et al. 2012a). In addition, to strengthen the translation of neuroPK parameters to patients, it is critical to investigate the CNS exposure in animal models mimicking the diseases of interest, and if possible in patients.

Issues related to the disequilibrium of drug concentrations at the BBB make it difficult to rank the importance of the PK parameters for the translation. Consequently, it is critical to assess the extent of human BBB transport and evaluate the potential impact of the degree of asymmetry on brain exposure in relation to target engagement or pharmacological activity early in drug discovery and development programs. The main reason for the observed asymmetry in BBB equilibration is the species-specific presence of efflux and influx transporters (see Chap. 4 for an overview of BBB transporters and pharmacoproteomics). There is no doubt that P-gp is one of the most important efflux transporters at the BBB (Tsuji et al. 1992, 1993; Terasaki and Hosoya 1999; Demeule et al. 2002; Mizuno et al. 2003; Lin 2004; Syvanen et al. 2008; Kodaira et al. 2011; Uchida et al. 2011a; Agarwal et al. 2012). However, the relative importance of P-gp in humans and rats was questioned after the breast cancer resistance protein (BCRP) was found to be the most abundant protein expressed in the human BBB (Uchida et al. 2011b). Nonetheless, cell lines transfected with human transporters, mostly only P-pg, are often used in lead optimization and candidate selection in the preclinical phases of drug discovery (see Chaps. 8 and 9 for a comprehensive overview of cell culture models of the BBB). Transporter knockout animals or chemically "knocked-out" animals (i.e., after the administration of P-gp or BCRP inhibitors) are used in drug discovery projects (see Chap. 10 for an exploration of in situ and in vivo animal models and Chap. 15 for the current thinking on this topic in the drug industry). Regardless of the "solid" status of in vitro and in vivo P-gp assays in drug discovery, both the rationale of the applied methods and the interpretation of the obtained results are debatable. Overall, it remains challenging to predict the BBB net flux of potential transporter substrates from rodent data. Consequently, due to the lack of translational knowledge, the recommendation not to advance efflux transporter substrates is often promoted in the drug industry (Di et al. 2012a).

The most critical issue in the assessment of brain exposure is related to using methods that can be applied for the same purpose across species including humans. In this regard, PET (see Chap. 11) has multiple advantages and is so far the most reliable technique that could be applied for translational purpose (Syvanen et al. 2009; Bauer et al. 2012; Wanek et al. 2013). The biggest challenge with PET is associated with the fact that total radioactivity is measured in both brain and blood. There are a few attempts to apply correction to total brain concentration obtained via PET using both $f_{u,brain}$ and $V_{u,brain}$ for the assessment of unbound cerebral concentrations (Gunn et al. 2012; Schou et al. 2015). Combined PET and brain microdialysis study design allows better understanding of the relationship between unbound and total concentrations and the convertibility between the methods (Gustafsson et al. 2017). In spite of multiple advantages, PET is considered too elaborate and expensive for screening purposes, and, hence, it is used only in later stages of drug development.

13.7 Current Status and Future Directions

Notwithstanding the immense progress in the understanding of drug delivery to the brain and improved screening cascades in drug discovery programs, the clinical success rate for novel neurotherapeutics is exceptionally low at present (Butlen-Ducuing et al. 2016; Cummings et al. 2016; Mehta et al. 2017; Danon et al. 2019). The approaches to the selection and optimization of compounds with sufficient delivery to the brain in drug discovery are currently stereotypical, high-throughput methods in most pharmaceutical companies. The complications associated with the measurement of active-site concentrations for potential CNS drugs have made surrogate methodologies (such as the assessment of brain ISF drug concentrations using matrices such as CSF and plasma) popular. There have been advancements in methodologies related to the assessment of $C_{u,brainISF}$, making it easier to measure the actual value rather than a surrogate. The use of the CSF as a relevant surrogate for $C_{u,brainISF}$ has been extensively investigated to support the rationale of its use in drug discovery (de Lange and Danhof 2002; de Lange et al. 2005; Liu et al. 2006, 2009a; Lin 2008; Friden et al. 2009b; Di et al. 2012a; de Lange 2013b; Loryan et al. 2014; Yamamoto et al. 2017; Ketharanathan et al. 2019; Vendel et al. 2019). Issues related to the sampling of CSF and interpretations of the data are discussed in Chaps. 4 and 15. However, despite the progress made, problems related to the veracity of the predicted values remain. It is important to remember that "you get what you measure" (Elebring et al. 2012), meaning that the definitions of the parameters and/or appropriate surrogates including critical interpretation are crucial. In this regard, understanding brain regional drug disposition in health and pathological conditions will require more focused investigations in the future, in order to improve the translational value of neuroPK parameters of NCEs.

The establishment of a PKPD relationship very early in drug development is a great advance for drug discovery (Chap. 15) (Bostrom et al. 2006; Westerhout et al. 2011; Stevens et al. 2012). Strategies to minimize neurotoxicity for non-CNS compounds are also of great interest (Wager et al. 2012). The exploration of the potential of mathematical modeling, particularly physiologically based PKPD modeling in drug discovery programs, will facilitate better understanding of the BBB transport of small molecules (Yamamoto et al. 2017; Vendel et al. 2019).

The role of the efflux and influx transporters and their potential interactions require investigation to provide further insight into active BBB transport supported by disease-specific BBB transcriptome and proteome atlases. We need to learn how to incorporate our knowledge on BBB cellular proteomics into drug transport mathematical modeling. Advancements in our understanding of pathological conditions (Part V) and their influences on the most important neuroPK parameters ($K_{p,uu,brain}$, $V_{u,brain}$, and $K_{p,uu,cell}$) will also improve the translational aspects of drug discovery.

13.8 Points for Discussion (Questions)

- What are the conceptual differences between the brain homogenate and brain slice methods?
- What allows the combination of the brain homogenate and brain slice methods to approximate the cellular unbound drug partitioning coefficient?
- Discuss the driving forces of BBB and CB drug transport.
- What is the physiological basis and pharmacokinetic impact of lysosomotropism for basic compounds?
- How can information about whether the compound is lysosomotropic influence the drug discovery and development processes?
- What are the pros and cons of using cut-off values for $K_{p,uu,brain}$ in relation to the evaluation of drug target engagement?
- Which neuroPK parameters are critical for translational medicine?
- Discuss the impact of the threefold difference cut-off point for methods in drug discovery.
- In which phase of drug discovery is it best to investigate the BBB transport and brain drug distribution of NCEs?
- What changes in neuroPK in particular diseases?

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