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P-glycoprotein Inhibition as a Strategy to Increase Drug Delivery across the Blood-Brain Barrier: Focus on Antidepressants

Thesis presented by

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Declaration

This thesis comprises original work carried out by the author and has not been submitted for any other degree at University College Cork, or elsewhere.

Author Contributions

All of the work described herein was performed independently by the author, with the following exceptions:

Chapter 2

Dr Gerard Clarke assisted with the development and running of the HPLC method.

Chapter 4, 5 and 6

Dr Richard O'Connor conducted the tail suspension tests and locomotor activity tests.

Chapter 5

Dr Gerard Clarke performed the analysis of monoamine content in brain tissue. Maria Donovan assisted with the microdialysis experiments.

Chapter 6

Dr Karen Scott carried out the majority of the RNA extractions. Gerard Moloney performed the majority of the PCR analyses.

Signed,

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Fionn E. O'Brien

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Abstract

Depression is among the leading causes of disability worldwide, affecting more than 350 million people globally. Currently available antidepressant drugs have unsatisfactory efficacy, with up to 60% of depressed patients failing to respond adequately to treatment. Emerging evidence has highlighted a potential role for the multidrug efflux transporter P-glycoprotein (P-gp; encoded by the *ABCB1* gene), expressed at the blood-brain barrier (BBB), in the aetiology of treatment-resistant depression. In the research outlined in this thesis, the potential of P-gp inhibition as a strategy to enhance the brain distribution and pharmacodynamic effects of antidepressant drugs was investigated in preclinical studies.

Microdialysis-based pharmacokinetic studies revealed that administration of the P-gp inhibitors verapamil or cyclosporin A (CsA) enhanced the BBB transport of the antidepressants imipramine and escitalopram in the conscious, freely moving rat. Furthermore, P-gp inhibition by verapamil increased brain concentrations of escitalopram following chronic administration in mice.

In addition to these *in vivo* studies, both imipramine and escitalopram were found to be transported substrates of human P-gp using an *in vitro* bidirectional transport model in Madine-Darby canine kidney cells transfected with *ABCB1* (MDCK-MDR1). These data indicate that *in vivo* findings demonstrating that P-gp limits the brain levels of these two antidepressants in rodents may translate to humans. Moreover, using the same *in vitro* model, we found that human P-gp exerted no effect on the transport of four other antidepressants: amitriptyline, duloxetine, fluoxetine and mirtazapine.

To build on the above-mentioned pharmacokinetic studies, the effect of P-gp inhibition on pharmacodynamic responses to antidepressant treatment was investigated *in vivo*. Pre-treatment with verapamil augmented the behavioural effects of escitalopram in the tail suspension test (TST), a widely used and well-validated model to assess antidepressant-like activity in mice. However, similar augmentation effects in the TST were not observed in studies involving CsA.

Moreover, pre-treatment with CsA exacerbated the behavioural manifestation of an escitalopram-induced mouse model of serotonin syndrome, a serious adverse reaction associated with serotonergic drugs. This finding highlights the potential for unwanted side-effects which may occur as a result of increasing brain levels of antidepressants by P-gp inhibition, although further studies are needed to fully elucidate the mechanism(s) at play.

Taken together, the research outlined in this thesis demonstrates that P-gp may restrict brain concentrations of escitalopram and imipramine in patients. Furthermore, we have shown that chronic treatment with a P-gp inhibitor and a P-gp substrate antidepressant results in elevated brain levels of the antidepressant. Moreover, increasing the brain distribution of an antidepressant by P-gp inhibition can result in an augmentation of antidepressant-like activity *in vivo*. These findings raise the possibility that P-gp inhibition may represent a potentially beneficial strategy to augment antidepressant treatment in clinical practice. Further studies are now warranted to evaluate the safety and efficacy of this approach.

Chapter 1:
General Introduction

1.1. Central nervous system barriers

The central nervous system (CNS), consisting of the brain and spinal cord, acts as the main processing centre for the entire nervous system. The neurons in the CNS communicate with each other via chemical and electrical signals which involve the movement of small ionic molecules across neuronal membranes. Thus, it is crucial that the ionic composition of the extracellular fluid in the CNS, which bathes the central neurons, is strictly controlled and regulated to facilitate precise intercellular signalling (Abbott, 2013). In contrast, the ionic composition of the blood varies considerably; for example, following food intake or exercise. It is therefore important that ion transport into and out of the CNS is tightly controlled to maintain ion homeostasis for optimal neuronal signalling. This is achieved by a number of CNS barriers, including the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB), which separate the CNS from the periphery (Figure 1.1). In addition to their contribution to ion homeostasis, the CNS barriers serve several additional functions, as outlined in Table 1.1.

Table 1.1: Functions of CNS barriers (adapted from Abbott, 2013)

Functions of central nervous system barriers
Maintenance of ion homeostasis, which is essential for optimal neural signalling
Regulation of molecular traffic, thereby ensuring sufficient uptake of essential nutrients and clearance of metabolic waste products
Protection of the CNS from toxins
Maintenance of a low protein environment in the CNS, thereby limiting proliferation and preserving neural connectivity
Separation of central and peripheral neurotransmitter pools, thereby limiting cross-talk and facilitating non-synaptic signalling in the CNS
Facilitation of immune surveillance while guarding against inflammation and resultant cell damage

The BBB, discussed in more detail in Section 1.2, exists at the level of the endothelial cells which line the blood microvessels in the brain, and is the most important interface for molecular flux between the blood and the brain (Abbott *et al.*, 2010). The BCSFB consists of the epithelium of the choroid plexus, which is the modified ependymal lining of the ventricles in the brain which secretes CSF (Laterra *et al.*, 1999b). Some solutes and drugs enter the brain primarily across the BCSFB, while others enter via both the BBB and BCSFB (Abbott, 2013). However, the majority of solutes and drugs gain entry to the brain via the BBB. The arachnoid barrier is another CNS barrier existing at the arachnoid epithelium in the middle layer of the meninges, which form the outer covering of the CNS. The arachnoid barrier plays a very limited role in the entry of solutes into the brain (Abbott, 2013).

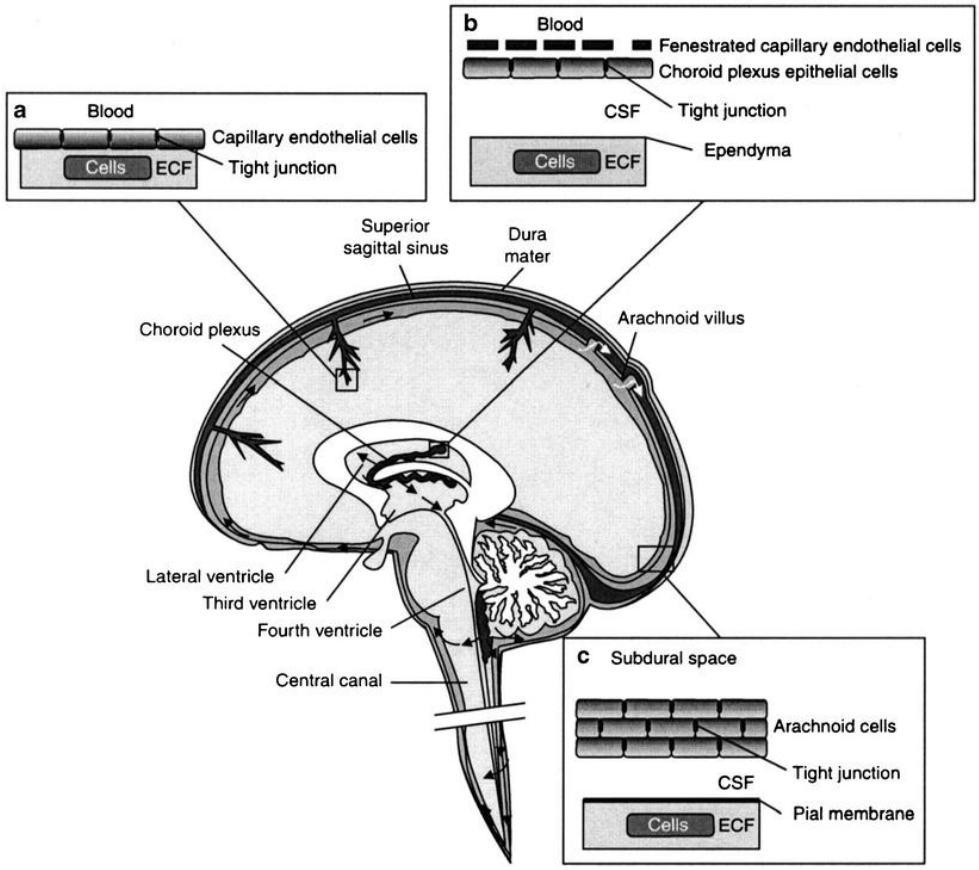


Figure 1.1: Location of the CNS barriers (Abbott, 2013)

1.2. The blood-brain barrier: a major obstacle in the delivery of drugs to the brain

The BBB plays a key role in maintaining homeostasis within the CNS; preserving the composition of the internal milieu despite variations in the periphery and protecting the brain against toxins, bacteria and viruses (Figure 1.2A). In addition, the BBB regulates the uptake of endogenous molecules and xenobiotics into the brain (Pardridge, 2007a; Neuwelt *et al.*, 2008; Pariante, 2008; Zlokovic, 2008; Abbott *et al.*, 2010; Neuwelt *et al.*, 2011).

Drug delivery is one of the major hurdles in the development of novel therapeutics for brain disorders (Pardridge, 2005). While alternative approaches exist to achieve direct drug delivery to the brain, there are severe disadvantages associated with such approaches (discussed in more detail in Section 1.2.4.1). As a result, most centrally acting drugs are administered peripherally (usually either orally or parenterally). Therefore, transport across the blood-brain barrier (BBB) needs to be achieved for these drugs to reach therapeutic concentrations at their site of action in the brain. The extensive network of blood capillaries in the brain, with an average of 40 µm between microvessels, means that, once drugs cross the BBB, the distances over which diffusion needs to take place to reach target cells are short (Pardridge, 2010). Therefore, in order to achieve widespread drug delivery throughout the brain, drug delivery across the BBB is the preferred route of drug transport.

Crucially, over 98% of small molecule drugs with potential for treatment of CNS disorders are unable to cross the BBB to reach target sites within the brain, while practically no large molecule therapeutics are able to penetrate the BBB (Figure 1.2B) (Pardridge, 2005; Neuwelt *et al.*, 2008). This means that many CNS disorders remain undertreated, even those with well-understood pathophysiology resulting in clearly defined therapeutic targets, such as Huntington's disease (Figure 1.2C). Moreover, emerging evidence indicates that certain small molecule drugs used clinically in the management of so-called "treatable" CNS disorders, such as depression and epilepsy, may not reach adequate concentrations in the brains of

some patients, thereby potentially contributing to the high prevalence of treatment failure in these diseases (Loscher and Potschka, 2005b; Uhr *et al.*, 2008).

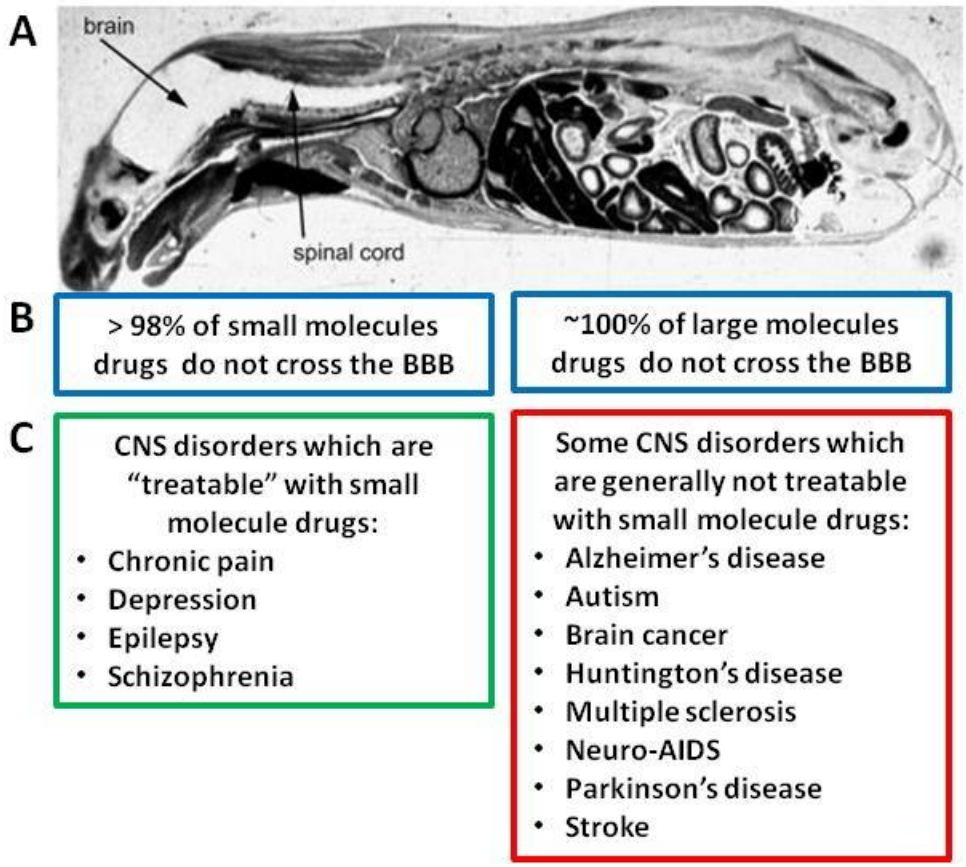


Figure 1.2: The BBB - a bottleneck in drug development (adapted from Pardridge, 2005). *A. Whole body autoradiogram of a mouse demonstrating that radiolabelled histamine penetrates all parts of the body apart from the CNS due to the CNS barriers. B. Statistics revealing the scale of the challenge presented by the BBB in the delivery of drugs to the brain. C. Only 5% of small molecule drugs are for the treatment of CNS disorders, and these are aimed at only four therapeutic indications.*

1.2.1. Structure and function of the BBB

The BBB consists of three main 'barriers' which regulate molecular flux between the blood and the brain. The 'physical barrier' consists of the tight junctions formed between the brain capillary endothelial cells (BCECs) lining the blood capillaries in the brain, which form the fundamental building block of the BBB (Figure 1.3).

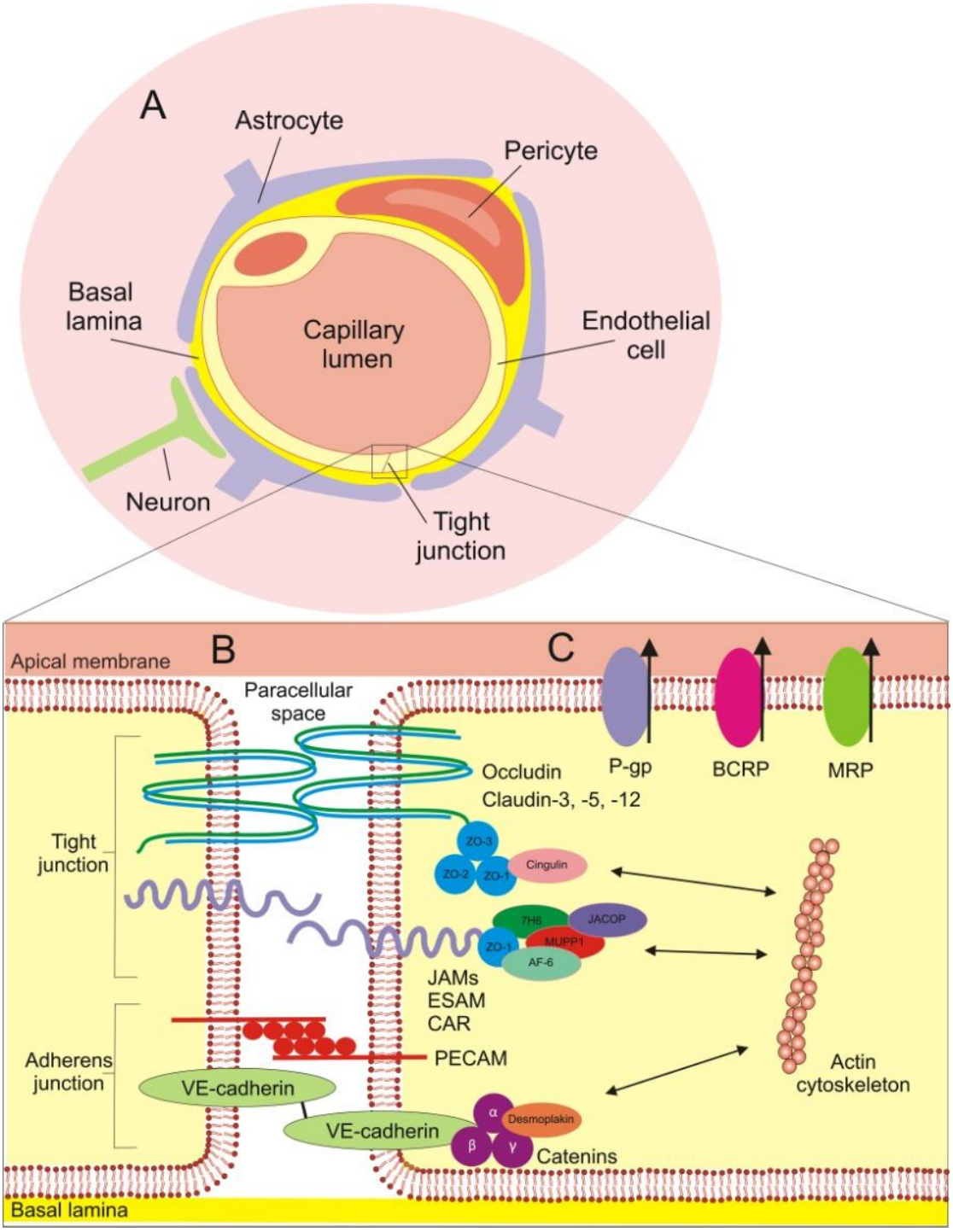


Figure 1.3: The blood-brain barrier (O'Brien *et al.*, 2012b). *A. The BBB consists of endothelial cells in the cerebral capillaries which, together with closely associated perivascular elements, such as pericytes, astrocyte endfoot processes and neurons, form the neurovascular unit. B. Illustration of tight junctions at the BBB C. Several drug efflux pumps, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins (MRP), are expressed at the BBB.*

Interactions between BCECs and other components of the neurovascular unit (Hawkins and Davis, 2005), which consists of BCECs and perivascular cells such as astrocytes, pericytes and neurons, induce the BBB-specific properties of the BCECs (Janzer and Raff, 1987; Hayashi *et al.*, 1997; Abbott *et al.*, 2006; Armulik *et al.*, 2010). In addition, the dynamic permeability of the BBB varies in response to intra- and intercellular signalling among these cells (Neuwelt *et al.*, 2011).

One of the key characteristics of the BBB is the formation of tight junctions between endothelial cells (Reese and Karnovsky, 1967; Brightman and Reese, 1969). These tight intercellular junctions consist of a highly complex network of transmembrane and intracellular linking proteins, as well as signalling and regulatory proteins (Figure 1.3B) (Wolburg and Lippoldt, 2002; Ballabh *et al.*, 2004; Wolburg *et al.*, 2009; Abbott *et al.*, 2010). Transmembrane proteins, such as occludin, claudin-3,-5 and -12 and three members of the immunoglobulin superfamily, junctional adhesion molecules (JAMs), endothelial selective adhesion molecules (ESAM) and the coxsackie- and adenovirus receptor (CAR), tightly link the endothelial cells together. Transmembrane proteins are linked to the actin cytoskeleton within the cell via first- and second-order cytoplasmic accessory scaffold molecules. Important first-order adaptor components, which bind directly to the transmembrane proteins, include the zona occludens proteins (ZO 1-3), while cingulin and junction associated coiled-coil protein (JACOP) are examples of second order adaptor proteins. Signalling and regulatory molecules include multi-PDZ-protein 1 (MUPP1), afadin (AF6) and 7H6. The adherens junction lends structural support to the endothelial tissue. It is made up of vascular endothelial cadherin (VE-cadherin), with the scaffolding proteins α-, β- and γ-catenin and desmoplakin, and the platelet-endothelial cell adhesion molecule (PECAM), which mediates homophilic adhesion.

The tight junctions between BCECs result in a continuous cellular wall, thereby effectively sealing the paracellular route of transport, which is important for hydrophilic or polar molecules which cannot penetrate the lipid environment of the cell membrane (Zlokovic, 2008; Abbott *et al.*, 2010). Thus, molecules must predominantly traverse the BBB via the transcellular route to gain access to the

brain (discussed in more detail in Section 1.2.2). However, a lack of fenestrations and low levels of transcytosis result in relatively low levels of transcellular transport at the BBB (Hawkins and Davis, 2005). Therefore, in general, physicochemical properties such as lipophilicity, molecular weight and charge determine the ability of a compound to cross the BBB, with only low molecular weight, lipophilic molecules able to penetrate the physical barrier presented by the BCECs.

In addition to the 'physical barrier' presented by the tight junctions between BCECs, there is also an 'enzymatic barrier' present at the BBB. Many drug metabolizing enzymes, including members of the cytochrome P450 (CYP) family and others such as glutathione-S-transferase and UDP-glucuronosyltransferase, are localized at the BBB (Ghersi-Egea *et al.*, 1994; Bauer *et al.*, 2008; Dauchy *et al.*, 2008; Dutheil *et al.*, 2009; Eyal *et al.*, 2009). However, it is worth noting that the profile of CYP450 enzymes present at the BBB differs considerably to that of the liver. CYP1B1 has been reported to be the most prominent CYP450 enzyme at the BBB, whereas CYPs 3A4, 2C9 or 2D6, involved in the hepatic metabolism of roughly 50% of drugs, were not detected at the BBB (Dauchy *et al.*, 2008). Nonetheless, compounds which are substrates of the BBB enzymes may be broken down as they traverse BCECs, thereby resulting in reduced bioavailability in the brain.

Furthermore, several uptake and efflux transporter proteins, expressed at the luminal and/or abluminal membranes of the BCECs, play a vital role in the regulation of molecule transfer across the BBB (Figure 1.3C) (Pardridge, 2007a). From a drug delivery perspective, the expression of drug efflux transporters at the BBB can limit brain exposure to substrate drugs, thereby constituting an 'efflux barrier' (Urquhart and Kim, 2009). Therefore, the brain permeability of certain compounds, which are subject to metabolism and/or drug efflux at the BBB, is much lower than would be predicted based on physicochemical properties alone (Cordoncardo *et al.*, 1989; Begley, 2004; Hermann and Bassetti, 2007).

1.2.2. Routes of transport across the BBB

The potential routes of drug transport across the BBB can be classified into three broad categories: (1) passive transport; (2) carrier-mediated transport; and (3) receptor-mediated transport (Figure 1.4).

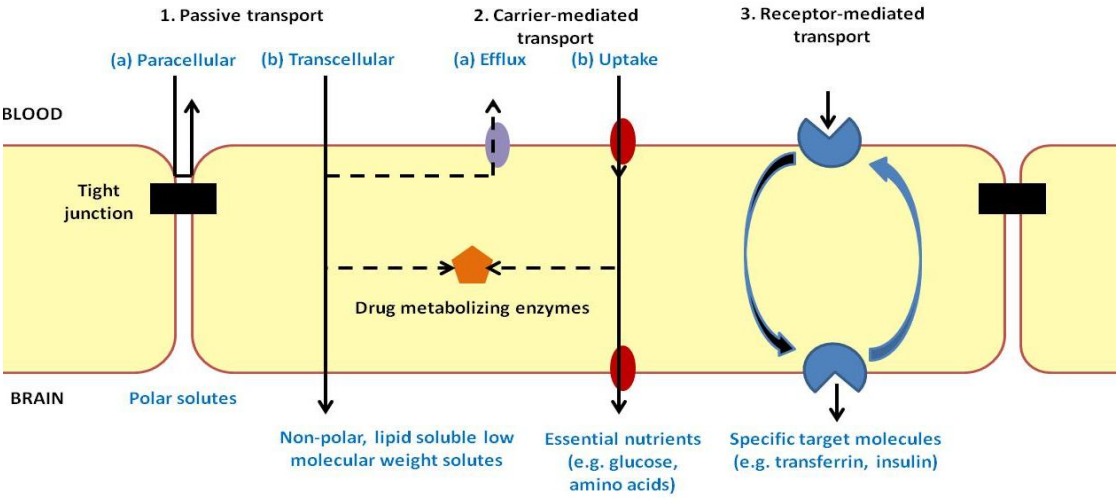


Figure 1.4: Routes of drug transport across the BBB. *1. Passive transport: (a) The paracellular route of transport, important for polar solutes, is effectively sealed at the BBB due to the presence of tight junctions between cells. (b) Low molecular weight, non-polar, unionized, lipophilic solutes can traverse the BBB via the transcellular route of passive transport. 2. Carrier-mediated transport: (a) Efflux transporters expressed at the BBB can limit the permeability of solutes availing of the transcellular route of transport by actively extruding them back against a concentration gradient. (b) Influx transporters facilitate the uptake of essential solutes, such as glucose, which otherwise would not reach the brain. 3. Receptor-mediated transport: Transport receptors, such as the transferrin receptor, expressed at the BBB bind to specific substrates, such as transferrin. The receptor-substrate complex is internalised within the cell and trafficked to the opposite membrane, where the substrate is released. The receptor is subsequently recycled to its original membrane.*

Passive transport involves drug diffusion down a concentration gradient, from a region of high concentration (i.e. blood) to lower concentration (i.e. brain tissue), and therefore does not require an energy source. It can be sub-divided into paracellular (i.e. between cells) and transcellular (i.e. through cells) transport.

In general, the paracellular route of transport is important for low molecular weight, hydrophilic, polar molecules which cannot traverse the lipid environment of

the cell membrane. However, owing to the aforementioned tight junctions at the BBB, under normal, healthy physiological conditions, the paracellular route of transport is effectively sealed at the BBB. Therefore, polar drug molecules are, in general, unable to cross the BBB and are not suitable candidates for the treatment of CNS disorders as a result (Pardridge, 2005; Abbott, 2013).

In contrast, small molecular weight, non-polar, lipophilic drug molecules can exploit the transcellular route of transport across the BBB, but only when they are in their unbound free state (i.e. not bound by plasma proteins). Such drug molecules must exhibit sufficient lipophilicity to be able to pass through the lipid bilayer and must typically be less than 400-500 Da in size to avoid steric hindrance (Pardridge, 2005). Thus, in general, low molecular weight, lipophilic drugs represent the category of drug best able to reach the brain (Figure 1.5).

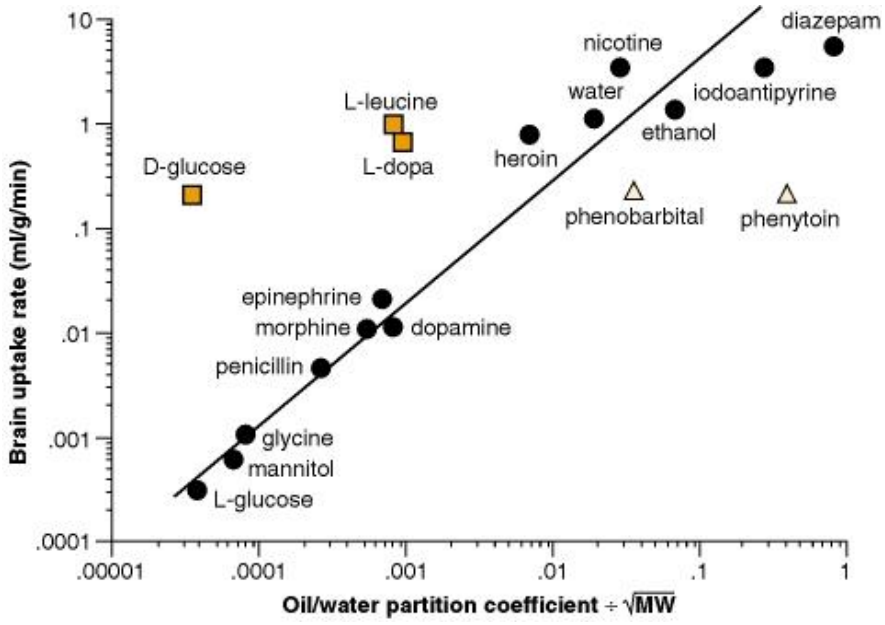


Figure 1.5: Relationship between lipid solubility, corrected for differences in molecular weight, and brain uptake rate of selected compounds (Laterra *et al.*, 1999a). *Higher values for oil/water partition coefficient correspond to greater lipophilicity. In general, brain uptake increases with increasing lipophilicity (compounds represented by black circles). Exceptions include substrates of active uptake processes (represented by orange squares), which exhibit greater uptake than would be predicted based on lipophilicity alone and substrates of efflux transporters or compounds which are highly plasma protein bound (represented by white triangles), which display lower uptake than would be predicted based on lipophilicity alone.*

However, low molecular weight lipophilic drug candidates are also susceptible to active extrusion by efflux pumps (discussed in more detail in Section 1.2.3) and enzymatic breakdown at the BBB, which could therefore restrict their brain bioavailability.

In addition to active efflux processes, several active carrier-mediated uptake or influx transporters are also expressed at the BBB (Table 1.2). Essential nutrients such as glucose, amino acids, amines, nucleosides, monocarboxylates and small peptides avail of this route of transport (Laterra *et al.*, 1999a). As a result, an adequate supply of these molecules, which otherwise would not cross the BBB, is maintained in the brain. Some therapeutic compounds, such as L-DOPA (the precursor of dopamine used in the treatment of Parkinson's disease) via the large neutral amino acid-transport system, gain access to the brain by exploiting naturally occurring carrier-mediated transport systems at the BBB (Laterra *et al.*, 1999a).

Most proteins are unable to cross the BBB due to their large size and hydrophilicity. As a result, concentrations of plasma proteins in the brain are very low. However, specific receptor-mediated transport processes exist at the BBB to facilitate the transcytosis of particular proteins, such as insulin and transferrin. Efforts have been made to exploit receptor-mediated transcytosis to enhance the delivery of peptide-based therapeutics to the brain, known as the 'molecular Trojan horse approach' (Pardridge, 2007a).

Table 1.2: Transport systems that operate from blood to brain (adapted from Laterra *et al.*, 1999a)

Transport System	Typical substrate
Metabolic substrates	
Hexose	Glucose
Monocarboxylic acid	Lactate
Large neutral amino acid	Phenylalanine
Basic amino acid	Lysine
Acidic amino acid	Glutamate
β-amino acid	Taurine
Amine	Choline
Purine	Adenine
Nucleoside	Adenosine
Saturated fatty acid	Octanoate
Vitamins and co-factors	
Thiamine	Thiamine
Pantothenic acid	Pantothenic acid
Biotin	Biotin
Vitamin B_6	Pyridoxal
Riboflavin	Riboflavin
Niacinamine	Niacinamine
Carnitine	Carnitine
Inositol	myo-Inositol
Electrolytes	
Sodium	Sodium
Potassium	Potassium
Chloride	Chloride
Hormones	
Thyroid hormones	T_3
Vasopressin	Arginine vasopressin
Insulin	Insulin
Other peptides	
Transferrin	Transferrin
Enkephalins	Leu-enkephalin

1.2.3. Drug efflux transporters at the BBB

As mentioned in Section 1.2.2, several drug transporters are expressed at the BBB. Efflux transporters from the adenosine triphosphate binding-cassette (ABC) superfamily, in particular, exert significant functional transport at the BBB. ABC transporters are primary active transporters, which use adenosine triphosphate (ATP) hydrolysis to power the active efflux of their substrates against concentration gradients. Forty-nine members of the ABC superfamily have been described in humans, and these are divided in to 7 major sub-families: ABCA to ABCG (Sharom, 2008).

ABC transporters are expressed at various sites within the body, including the kidney, liver, intestine and adrenal gland. Importantly, several members of the ABC superfamily are localized at the BBB (Figure 1.6); including P-glycoprotein (P-gp; encoded by the multidrug resistance 1 (*MDR1*/*ABCB1*) gene), breast cancer resistance protein (BCRP; encoded by *ABCG2*) and multidrug resistance-associated proteins 4 and 5 (MRP4 and 5; encoded by *ABCC4* and *5* respectively) (Begley, 2004; Loscher and Potschka, 2005a; Nies, 2007; Dauchy *et al.*, 2008). The functional impact of these ABC transporters on the brain accumulation of several drugs has been elucidated in preclinical studies involving knockout mice and specific inhibitors (de Vries *et al.*, 2007). There is significant overlap in substrate specificity between P-gp, BCRP and the MRPs, and many commonly used drugs are included among their substrates (de Vries *et al.*, 2007; Sharom, 2008; Zhou, 2008). Interestingly, P-gp and BCRP appear to work in concert to synergistically limit the brain penetration of certain drugs, including the anti-cancer agents topotecan and lapatinib (de Vries *et al.*, 2007; Polli *et al.*, 2009).

Furthermore, ABC transporters have been implicated in resistance to pharmacotherapy, most notably in oncology, where the expression of these efflux pumps by cancer cells may confer resistance to chemotherapy with cytotoxic drugs in cancer patients (Gottesman *et al.*, 2002; Szakacs *et al.*, 2006). In addition, there is increasing evidence that ABC transporter-mediated drug efflux at the BBB may limit brain drug delivery of several CNS drugs (Loscher and Potschka, 2005b), thereby

leading to treatment failure in various brain disorders, including brain cancer (Pauwels *et al.*, 2007), epilepsy (Siddiqui *et al.*, 2003) and stroke (Spudich *et al.*, 2006). In the field of psychiatry, much recent attention had been given to the role of efflux pumps in the pharmacokinetic profile of antidepressant drugs. Increasing evidence suggests that P-gp, in particular, may limit the ability of several antidepressants to cross the BBB, thus resulting in inadequate brain concentrations and therefore contributing to the poor success rate of current antidepressant therapies (Uhr *et al.*, 2008). The role of P-gp in the contribution to treatment failure in CNS disorders is discussed in more detail in Section 1.3.5.

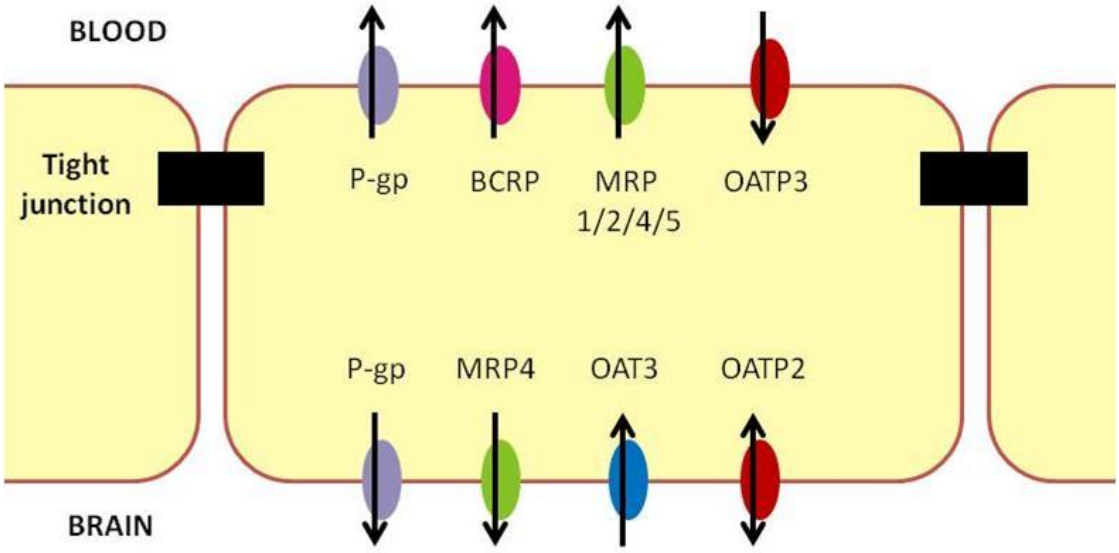


Figure 1.6: Drug transporters at the BBB. *Several drug efflux and uptake transporters are expressed at the BBB. P-gp, BCRP and the MRPs expressed at the apical (i.e. blood) membranes of BCECs restrict the transport of their substrates across the BBB. There is also limited evidence of P-gp expression at the basolateral (i.e. brain) membrane (Bendayan et al., 2006), although the functional relevance of this is unclear.*

1.2.4. Approaches to overcome the BBB to achieve drug delivery to the brain

Existing methods to enhance the brain delivery of drugs which cannot cross the BBB are largely inadequate for various reasons. They can be divided into two broad categories: invasive and non-invasive approaches.

1.2.4.1. Invasive approaches

Invasive transcranial drug delivery approaches involve bypassing the brain barriers to directly administer drugs to the brain, such as direct intracerebroventricular (ICV) injection or the intracerebral implantation of a controlled release drug delivery device (Pardridge, 2007b).

Severe limitations associated with these techniques, not least the requirement for surgical intervention, preclude their widespread use in clinical practice. In particular, transcranial drug delivery techniques fail to achieve adequate drug distribution throughout the brain due to the inefficiency of drug diffusion in brain tissue (Pardridge, 2005). While high local concentration are achieved at the site of injection or implantation, which can lead to toxicity, sufficient drug levels are not reached at more distant brain regions as drug concentrations decline exponentially with distance from the injection/implantation site (Pardridge, 2010). Attempts have been made to overcome this limitation by utilising convection-enhanced-diffusion, which involves the continuous infusion of fluid into the brain following implantation, but to limited effect (Salvatore *et al.*, 2006). Therefore, primarily due to the invasive nature of the approach and the problems associated with drug diffusion in brain tissue, direct transcranial drug administration only represents a viable strategy in particular circumstances, such as brain cancer, where the clinical benefit outweighs the risk involved.

1.2.4.2. Non-invasive approaches

Considering that the brain is served by an extensive network of blood capillaries, with typically only 40 µm between capillaries in the brain, it is ideal to deliver drugs intended for global brain distribution via the vascular route (Pardridge, 2010). This represents a challenge for therapeutics which are unable to cross the BBB. However, certain strategies exist to enhance the delivery of such drugs to the brain.

One potential approach to achieve this goal is by transient disruption of the BBB to facilitate drug delivery to the brain. This can be done by the intracarotid infusion of hyper-osmotic solutions, as described by Neuwelt and colleagues over 30 years ago (Neuwelt *et al.*, 1979; Neuwelt *et al.*, 1980). However, this strategy has been

rejected as impractical for general widespread use due to the obvious disadvantages associated with non-selective disruption of the important protective and homeostatic functions played by the BBB, including the inducement of epileptic type seizures (Marchi *et al.*, 2007). Future research, perhaps involving the use of RNA interference (Campbell *et al.*, 2011), localised ultrasound irradiation (Mesiwala *et al.*, 2002; Hynynen, 2008) or selective pharmacological modulation of BBB permeability (Carman *et al.*, 2011), may facilitate highly selective alteration of BBB function to enable the targeted delivery of drugs to the brain.

Alternatively, rather than disrupting the BBB *per se*, attempts have been made to enhance drug transport across the intact, fully functional BBB. These efforts have included altering drug molecules using medicinal chemistry approaches (Greig *et al.*, 1990), linking drug molecules to CNS-targeting moieties (the BBB molecular 'Trojan Horse' approach) (Pardridge, 2006) or the incorporation of drug molecules into colloidal carriers (Kreuter *et al.*, 2003). The incorporation of CNS drug molecules into colloidal drug carriers, thereby potentially masking unwanted physicochemical properties, protecting from enzymatic degradation or drug efflux and enhancing CNS bioavailability via increased transmembrane diffusion, represents a particularly promising novel approach to achieve drug delivery to the brain via the bloodstream without necessitating disruption of the BBB (Wohlfart *et al.*, 2011a).

1.2.5. Methods to predict or measure drug transport across the BBB

Various approaches, ranging from *in silico* to *in vitro* and *in vivo*, exist to assess drug transport across the BBB. Each of these approaches has associated strengths and weaknesses, as will be outlined in the forthcoming sections, and can be best employed at different stages in the drug discovery process.

1.2.5.1. In silico

In silico approaches to evaluate drug transport across the BBB make use of computer-based simulations to predict whether a compound will penetrate the BBB based on its molecular characteristics. In general, *in silico* approaches facilitate

relatively cheap and rapid high-throughput screening of the BBB permeability of potential lead compounds in early drug discovery, but typically only achieve ~70-80% prediction accuracy (Goodwin and Clark, 2005; Dureja and Madan, 2007; Broccatelli *et al.*, 2012).

These *in silico* models are based on relationships between BBB permeability, as measured experimentally *in vitro* or, preferably, *in vivo*, and molecular characteristics of the test compounds used. For example, early work in this field focused on the relationship between lipophilicity and BBB permeability (Oldendorf, 1974; Levin, 1980).

The utility of *in silico* models is primarily dependent on the quality and quantity of the training and test datasets on which the model is based and the molecular descriptors included in the model (Mensch *et al.*, 2009).

With regards to such datasets, the 'garbage in, garbage out' dictum applies (Mensch *et al.*, 2009). It is crucial that the *in vivo* data on which the model is based is appropriate and accurate, ideally generated under the same experimental conditions in the same laboratory. In addition, it is necessary to be aware of the limitations of the model in terms of its 'chemical space', as attempting to apply a model to make predictions to molecules from outside the chemical space of the dataset could result in misleading and inaccurate results (Goodwin and Clark, 2005).

Several molecular descriptors which influence BBB permeability have been identified. Since early studies reported links between lipophilicity and BBB permeability (Oldendorf, 1974; Levin, 1980), further key characteristics, such as molecular weight, the polar surface area and the number of hydrogen-bond acceptors, have come to light (Mensch *et al.*, 2009). However, predicting BBB permeability based on such physicochemical parameters alone focuses on passive transcellular transport only, and neglects carrier-mediated and enzymatic processes which may influence brain bioavailability. This is a major disadvantage of the majority of models. Increasing recent effort has focused on developing quantitative structure activity relationship (QSAR) models to predict if a compound will be subject to active transport processes (as discussed in section 1.3.7.1). Integration of

such QSAR information in more complex *in silico* models may facilitate more accurate prediction of BBB permeability in the future, but to date no *in silico* model which fully reflects the intricacies of the BBB exists (Mensch *et al.*, 2009).

1.2.5.2. In vitro

A valid, predictive and representative *in vitro* screening tool would be of enormous benefit in the development and design of novel approaches to overcome the BBB, and to predict the BBB permeability of novel drug compounds. Ideally, such a model would consist of a readily available, convenient, predictable and reproducible immortalized endothelial cell line, with brain endothelial cell characteristics, low paracellular permeability (due to the formation of tight junctions) and functional expression the various transporters and enzymes present at the BBB *in vivo* to physiologically relevant levels. However, no such ideal *in vitro* model has been developed as yet (Cecchelli *et al.*, 1999; Reichel *et al.*, 2003; Garberg *et al.*, 2005; Avdeef, 2011).

In vitro systems used to investigate drug permeability across the BBB can be divided into two broad categories: cells derived from a cerebral origin and cells not derived from a cerebral origin (Table 1.3).

Brain capillary endothelial cells (BCECs), the fundamental building block of the BBB, have been isolated with little or no contamination by other cell types (e.g. glial cells, pericytes etc) from a range of species, including mouse, rat, cow, sheep, pig monkey and human, and generally produce confluent cell monolayers after 9 days in culture. However, the isolation of BCECs for primary systems requires time-consuming, technically challenging and tedious work, and can be responsible for inter-lab variations (Reichel *et al.*, 2003). In addition, cultured BCECs lose or down-regulate many key characteristics of the BBB *in vivo*, such as tight junctional complexity, specific transporters and enzymes, especially when isolated from their natural milieu (Cecchelli *et al.*, 1999). This limitation can be somewhat overcome by co-culturing BCECs with astrocytes, however, other limitations still apply (Cecchelli *et al.*, 1999). Immortalised brain endothelial cell lines, which demonstrate high TEER values when cultured on transwell systems, have been recently developed

(Weksler *et al.*, 2005; Poller *et al.*, 2008). These systems may represent a useful investigational tool in the future, but further validation is needed before their predictive utility can be definitely evaluated.

Alternatively, cells derived from a non-BBB origin which form confluent monolayers with tight junctions when grown on an appropriate transwell support, can be used to determine drug permeability across a biological membrane which may reflect BBB permeability (Hellinger *et al.*, 2012). Furthermore, such non-cerebral cell lines may possess, or can be transfected with, certain properties which exist at the BBB, such as P-gp expression. Cell lines commonly used for this purpose include the naturally P-gp-expressing human colorectal adenocarcinoma (Caco-2) cells and *ABCB1*-transfected, P-gp expressing Madine-Darby canine kidney cells (MDCK-MDR1). These model cell-lines are polarized, with distinct apical and basolateral membrane domains, when cultured on an appropriate transwell support.

As P-gp is exclusively expressed on the apical membrane of the cultured cells, the comparison of apical-to-basolateral (A→B) permeability and basolateral-to-apical (B→A) permeability for a test compound indicates if it is a P-gp substrate (Lin, 2007). Furthermore, these cell systems can be used to determine a drug's apparent permeability (P_{app}) across a P-gp expressing membrane. However, the fact that these cells are from a non-cerebral origin is a considerable disadvantage, especially when one considers that membrane properties influence P-gp mediated transport (Romsicki and Sharom, 1999; Hellinger *et al.*, 2012). In addition, non-cerebral cells transfected with a singular aspect of the BBB, such as P-gp for example, fail to fully reflect the variety of complex active processes present at the BBB *in vivo*. Nonetheless, they can represent useful models for the prediction of BBB transport (Hellinger *et al.*, 2012).

Table 1.3: *In vitro* models to predict drug transport across the BBB (adapted from Reichel *et al.*, 2003; Garberg *et al.*, 2005; Mensch *et al.*, 2009)

Method	Advantages	Limitations
Cells derived from a cerebral origin		
Isolated brain capillaries / microvessels	• Can be isolated from various sources (animal/human) • Metabolically active • Reflective of *in vivo* situation	• Significant loss of ATP → significant loss of activity • Difficult to access luminal surface of microvessels, therefore can only conduct drug transport experiments from abluminal to luminal side • Thus unsuitable for BBB-permeability screening
Primary BCECs (monoculture)	• Close to *in vivo* situation • Maintain many BBB-specific *in vivo* features • Can be prepared from a variety of sources (bovine and porcine preferred due to higher yield than from rats; ethical and tissue access constraints with human sources)	• Some significant BBB-features down-regulated or lost during preparation • Tend to overestimate P_e values for lipid-mediated transport and underestimate P_e for carrier-mediated transport • Lose expression of BBB-specific proteins (e.g. drug transport pumps)
Primary BCECs (co-culture or astrocyte-conditioned medium)	• As per monoculture, plus: • Up-regulation of P-gp function and increased TEER relative to monocultures; therefore closer approximation of *in vivo* situation	• Time and resources required to isolate, seed and incubate primary BCECs and astrocytes • Poor reproducibility
Immortalized BCECs	• Avoid disadvantages of primary systems with respect to time, effort and reproducibility • Form monolayers • Useful for mechanistic and biochemical studies	• Do not generate complete tight junctions (to date), therefore unsuitable for studying BBB transport due to paracellular leakiness

Method	Advantages	Limitations
Cells derived from non-cerebral origin		
MDCK	• Reproducible, high TEER value • Low sucrose permeability • Easy to grow • Can be transfected with P-gp (to achieve polarized expression) • Most representative of *in vivo* permeability for passive and P-gp-effluxed compounds	• Does not express other BBB-specific proteins which may play significant roles in drug transport
Caco-2	• Well-established model for permeability studies (intestinal) • Naturally expressed P-gp in a polarised fashion when cultured on a transwell support	• Many differences from BCECs – morphology, protein expression, metabolism etc • Expression of a variety of other efflux and uptake transporters in addition to P-gp
P_e: apparent permeability; TEER: transepithelial electrical resistance (a measure of the tightness of junctions between cultured cells)		

1.2.5.3. In vivo and in situ approaches

Due to the limitations of the available *in silico* and *in vitro* BBB models, and the complexity of the processes ongoing at the BBB, *in vivo* models remain the best way of assessing the ability of a compound to cross the BBB. Moreover, *in vivo* approaches provide crucial reference information for the testing and validation of other models (Mensch *et al.*, 2009). Several different *in vivo* approaches to investigate drug transport across the BBB exist, including intravenous injection followed by decapitation and brain collection, the brain uptake index approach, brain perfusion, imaging studies and microdialysis. These approaches vary in terms of their sensitivity and usefulness (Bickel, 2005; Nicolazzo *et al.*, 2006; Mensch *et al.*, 2009).

Intravenous injection followed by decapitation and determination of drug concentration in brain tissue represents a relatively simple, sensitive and representative method of measuring brain uptake and has been described as the 'gold standard' for assessing BBB permeability (Nicolazzo *et al.*, 2006). However, using this approach, it is only possible to obtain brain concentrations at a terminal time-point (i.e. one time-point per animal) and it is therefore necessary to use a large number of animals to elucidate the brain distribution kinetics of a drug using this method. Furthermore, as the brain vasculature accounts for approximately 5% of the total brain volume (Wohlfart *et al.*, 2011b), it is necessary to consider the presence of drug within the brain capillaries which has not actually crossed the BBB but will appear in brain homogenate samples. Many studies fail to account for this issue, which in turn limits the conclusions that can be drawn from such studies in relation to the BBB penetration of the compound of interest.

The problem of drug present in the cerebral vasculature can be overcome to some extent by using the capillary depletion technique (Triguero *et al.*, 1990), which utilises density centrifugation to separate vascular components from other brain tissue. Thus it is possible to measure drug concentrations in the supernatant, which should theoretically only contain tissue from the brain parenchyma. However, capillary depletion is an imperfect technique. For example, drug bound to low

affinity or non-specific binding sites on the plasma membrane of brain capillary endothelial cells may dissociate during the centrifugation process, thus producing misleading results (Pardridge, 1995).

The brain uptake index (BUI) and isolated brain perfusion *in situ* techniques offer some advantages compared to the intravenous administration approach outlined above (Bickel, 2005). Both of these techniques specifically investigate processes ongoing at the BBB, thereby avoiding factors such as metabolism in other organs which can complicate deconvolution of data from intravenous studies. In addition, the composition of the intravascular fluid in the cerebral microvasculature can be readily manipulated, thereby facilitating investigation of the impact of factors such as test solute concentration, protein binding and osmotic pressure on BBB permeability (Bickel, 2005).

The BUI, first reported in the 1970s (Oldendorf, 1970) is a single pass technique, whereby the test compound (along with a reference compound which is impermeable to the brain as a control to correct for the fraction of the bolus remaining in the vascular lumen at the time of sampling) is rapidly injected into the common carotid artery of anaesthetised animals. Brain uptake is determined by sampling of brain tissue 5-15 sec after the injection. The short timeframe involved eliminates systemic recirculation and wash-out as potential confounding factors (Bickel, 2005). However, the single pass nature of the technique limits the utility of the BUI to substances with moderate-to-high BBB permeability, as the uptake of less rapidly permeable compounds may not be detected. Morphine, for example, has well-established CNS effects, but its BUI is scarcely above that of impermeant vascular markers (Bickel, 2005). In addition, the short timeframe involved precludes identification of slower uptake processes, such as receptor mediated endocytosis.

Some of these deficiencies can be overcome by adopting brain perfusion methods. These approaches typically involve catheterization of the carotid artery and disruption of endogenous blood flow to the brain, for example by severing the heart ventricles. An oxygenated physiological buffer containing the test compound is then perfused for a period of time up to 10 minutes (Bickel, 2005). Thus, the

identification of compounds taken up into the brain via receptor mediated transport, which takes place over a period of minutes rather than seconds, can be achieved using this technique (Bickel, 2005).

Both the BUI and brain perfusion *in situ* approaches involve the use of anaesthesia in the animals during the experiment, which may be a severe limitation given the potential effects of anaesthesia on BBB permeability (Saija *et al.*, 1989; Tetrault *et al.*, 2008; Thal *et al.*, 2012). In addition, the problems previously outlined in relation to drug contained within the brain vasculature also apply. The intracerebral microdialysis technique offers several advantages compared to these methods.

1.2.5.4. Intracerebral microdialysis

Intracerebral microdialysis facilitates the determination of unbound concentrations of both endogenous and exogenous substances in brain extracellular fluid (ECF) as a function of time in conscious, freely moving animals (de Lange *et al.*, 2000; Plock and Kloft, 2005; Anderzhanova and Wotjak, 2013). Derived from earlier attempts to sample amino acid and electrolytes in neuronal extracellular fluid across a semi-permeable membrane, using the 'push-pull' method, the modern-day intracerebral microdialysis technique was pioneered by Ungerstedt and co-workers at the Karolinska institute in Stockholm, Sweden in the 1970s and 1980 (Ungerstedt and Pycock, 1974; Darvesh *et al.*, 2011). While it has predominantly been used to monitor fluctuations in brain concentrations of neurotransmitters such as serotonin, dopamine and noradrenaline in response to various interventions (Ungerstedt and Hallstrom, 1987; Di Chiara *et al.*, 1996), it has also been used to monitor brain extracellular fluid levels of many drugs, including morphine (Groenendaal *et al.*, 2007), imipramine (Sato *et al.*, 1994) and buspirone (Tsai and Chen, 1997).

The key component of the microdialysis system is the microdialysis probe, which consists of a small semi-permeable hollow membrane connected to inlet and outlet tubing. The microdialysis probe can be surgically inserted into various body fluids or body tissues, including the brain. Moreover, the probe can be targeted to specific brain regions of interest with a high degree of accuracy using a stereotaxic frame.

While microdialysis probes can come in a variety of different designs (de Lange *et al.*, 2000), the most commonly used design in intracerebral studies is the concentric design (Figure 1.7A). At the microdialysis probe membrane, unbound molecules capable of passing through the semi-permeable membrane will diffuse across the membrane down their concentration gradients, either into or out of the probe lumen (Figure 1.7B). According to Fick's first and second laws (Fick, 1855), the rate of diffusion is directly proportional to the concentration gradient. Therefore, the amount of an analyte present in the dialysate will be proportional to the concentration in the periprobe fluid (Anderzhanova and Wotjak, 2013).

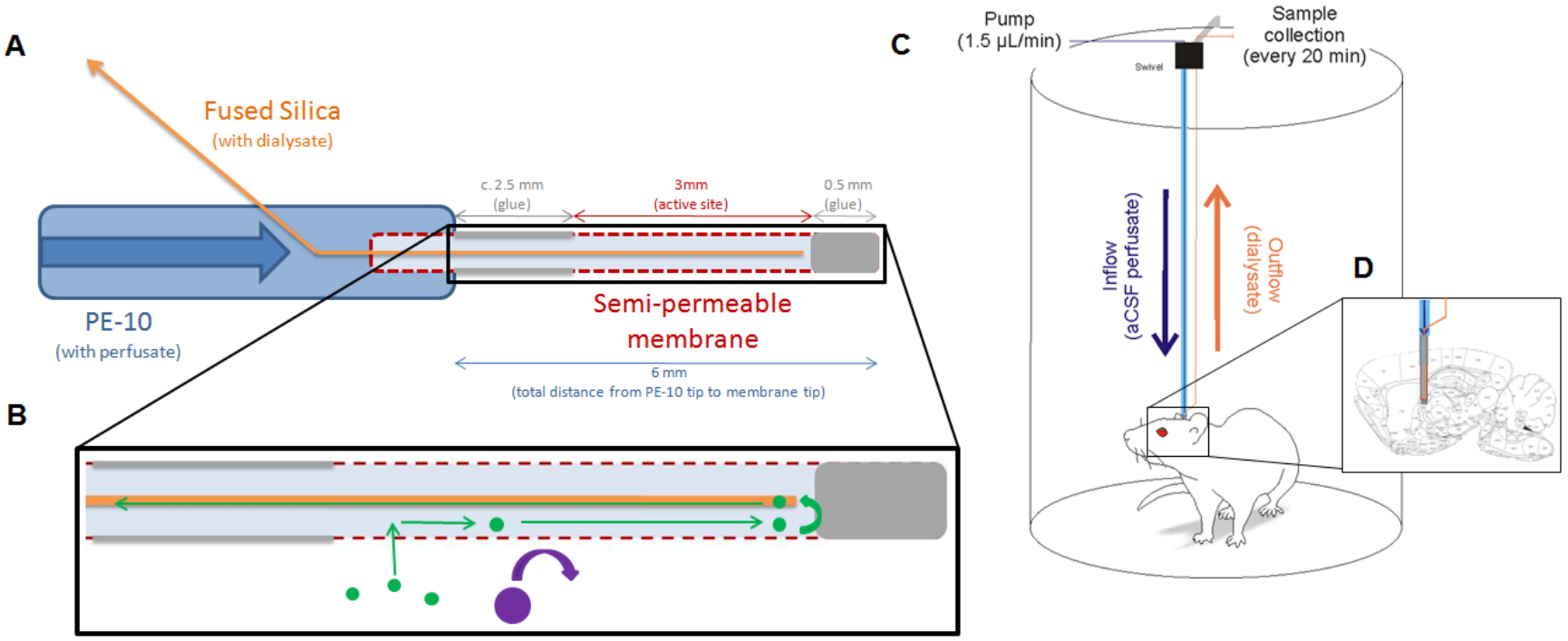


Figure 1.7: Intracerebral microdialysis. A *The microdialysis probe consists of inlet and outlet tubing, connected to a semipermeable membrane. The membrane length and the active area can be tailored to target specific brain regions of interest. The perfusate is continuously pumped through the probe at a slow flow rate B. Compounds with sufficiently low molecular weight (green circles) will diffuse into the probe down their concentration gradient, and be pumped out through the outlet tubing. C-D The microdialysis probe can be specifically targeted to a brain region of interest with a high degree of accuracy using a stereotaxic frame during implantation. Moreover, the probe remains intact in conscious, freely moving animals, thereby facilitating sampling without a need for anaesthesia.*

During a microdialysis experiment, the probe is continuously perfused with an aqueous solution, known as the perfusate, which mimics the ionic composition of the periprobe fluid, at a constant low flow rate (typically 0.5-5 $\mu l.min^{-1}$). Outlet tubing connects the membrane of the probe to the collection vial, in which the sample, known as the dialysate, is collected for analysis by a suitable analytical technique capable of dealing with the typically small volumes and low concentrations associated with microdialysis studies. The concentration of the analyte in the dialysate will reflect the concentration in the periprobe fluid. However, as microdialysis experiments are not conducted under equilibrium conditions, the dialysate concentration will always be lower than the actual concentration in the fluid surrounding the probe. The relationship between dialysate and periprobe concentrations is termed 'probe recovery' (Anderzhanova and Wotjak, 2013)

The probe recovery is dependent on many factors, including flow rate, membrane properties, perfusate composition, physicochemical properties of the analyte of interest and temperature (de Lange *et al.*, 2000). Probe recovery can be easily determined *in vitro* by immersing the probe in a well filled with a solution of known concentration of the analyte of interest, and then comparing dialysate concentrations to well concentrations. Determination of *in vivo* probe recovery is more difficult, but can be achieved by retrodialysis (where the molecule of interest is included in the perfusate in a naive animal and percentage loss is calculated) (Bouw and Hammarlund-Udenaes, 1998). A limitation of this approach is the assumption that drug diffusion will be equal in both directions (i.e. the percentage loss in retrodialysis experiments will be equal to the percentage gain in traditional microdialysis experiments). More sophisticated approaches to establish *in vivo* probe recovery, which do not make this assumption, include the no-net-flux (Lonnroth *et al.*, 1987) or the more complex dynamic-no-net-flux methods (Olson and Justice, 1993).

The no-net-flux methodologies involve perfusing different concentrations (C_{inlet}) of the compound of interest through the probe in a subject in which steady-state

levels of the drug have been achieved. The resulting dialysate concentrations (C_{outlet}) are measured, and the difference between C_{inlet} and resultant C_{outlet} are plotted as a function of C_{inlet} (Figure 1.8). This plot will cross the x-axis at the point where C_{inlet} is equal to the concentration in the periprobe fluid (as there has been no loss or gain of the compound). If the plot is linear, and if true steady-state conditions exist, the slope of the function will equal the *in vivo* recovery of the probe for that compound (de Lange *et al.*, 2000; Anderzhanova and Wotjak, 2013).

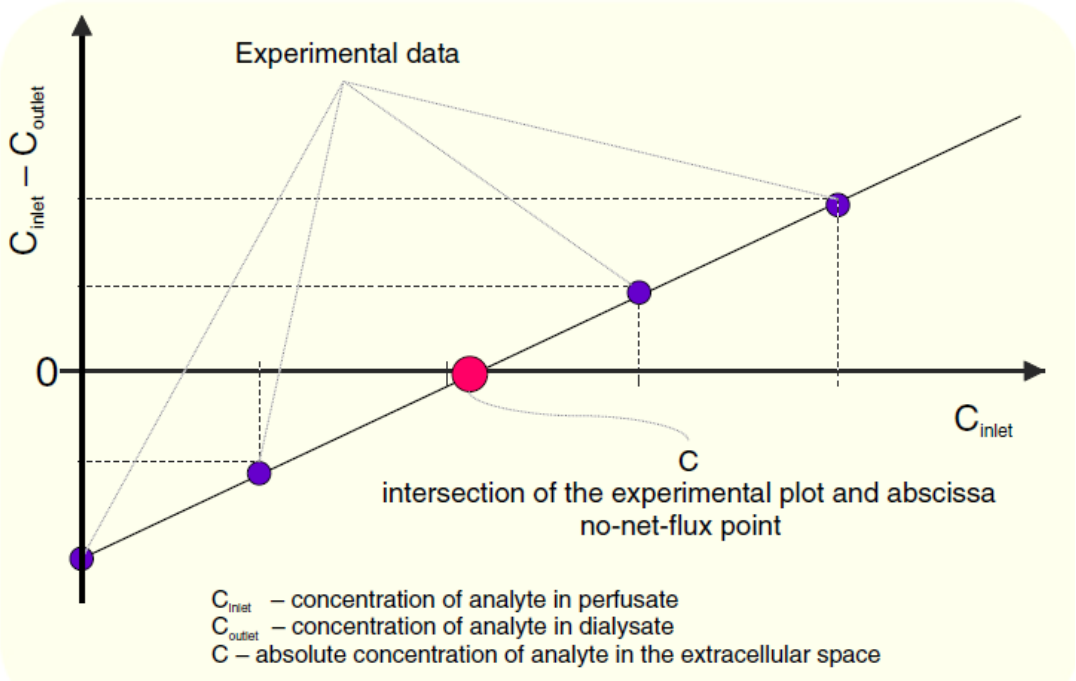


Figure 1.8: The principle of the no-net-flux method to determine *in vivo* microdialysis recovery (Anderzhanova and Wotjak, 2013). *Different concentrations of the compound of interest (C_{inlet}) are perfused through a microdialysis probe implanted in an animal in which steady state levels of the compound have been achieved and maintained. The concentration emerging from the probe (C_{outlet}) is determined, and the difference between the perfusate and dialysate concentrations (i.e. C_{inlet} - C_{outlet}) is plotted as a function of the perfusate concentration. The point at which the plot crosses the x-axis represents the point at which the perfusate concentration is equal to that of the periprobe fluid, and the slope of the line is equal to the in vivo receovery of the probe for that compound.*

While the use of these techniques to establish *in vivo* probe recovery facilitates the calculation of actual analyte concentrations in the brain ECF, there are several limitations and difficulties associated with them (de Lange *et al.*, 2000). For

example, they involve extremely time-consuming and challenging methodologies, and attempts to validly employ these techniques are often fraught with difficulties. To overcome these difficulties, a modified 'ultraslow' quantitative microdialysis technique has been recently developed (Cremers *et al.*, 2009). However, this approach is proprietary, and cost issues may preclude its use, particularly in academia. Therefore, in certain circumstances, it is on balance preferable to calibrate probes *in vitro* only, and to selectively use probes with similar rates of recovery to enable comparison of relative changes in dialysate concentrations between different animals without a need to extrapolate absolute brain ECF concentrations (Page and Lucki, 2002; Anderzhanova and Wotjak, 2013). While this approach does not yield quantitative results *per se*, it facilitates evaluation and comparison of relative levels of the analyte in different treatment groups.

Intracerebral microdialysis offers several key advantages over other approaches to determine BBB transport (de Lange *et al.*, 2000; Anderzhanova and Wotjak, 2013). Using microdialysis, it is possible to sample from the brain ECF repeatedly over time in the same animal, thus facilitating a high degree of temporal resolution unattainable with other methods. In addition, this serves to reduce the number of animals required to conduct a pharmacokinetic study, in accordance with the 'reduction' principal of the 3Rs in animal research (http://www.nc3rs.org.uk/). Moreover, the animal is both conscious and freely moving throughout the sampling period (Figure 1.7C), therefore avoiding potential effects of anaesthesia on BBB function. In addition, microdialysis samples only contain drug which has penetrated the BBB and is present in the brain ECF, as opposed to other techniques which determine the concentration of drug in brain tissue which includes cerebral capillaries. Furthermore, only unbound drug molecules will cross the microdialysis probe semipermeable membrane, and it is these free drug molecules which are pharmacologically active. Therefore, microdialysis facilitates the measurement of pharmacologically relevant active site concentrations of drug molecules in the brain (Hammarlund-Udenaes, 2010).

1.3. P-glycoprotein

P-glycoprotein (P-gp) was first identified in the 1970s, when it was discovered to confer resistance to cancer cells against chemotherapeutic agents *in vitro* (Juliano and Ling, 1976), and was subsequently found to be localized in blood capillaries in the brain (Cordoncardo *et al.*, 1989; Thiebaut *et al.*, 1989). P-gp is widely expressed at specific sites within the body, including the BBB (Figure 1.9). It is the most studied, and arguably most functionally important, member of the ABC family of drug efflux transporters.

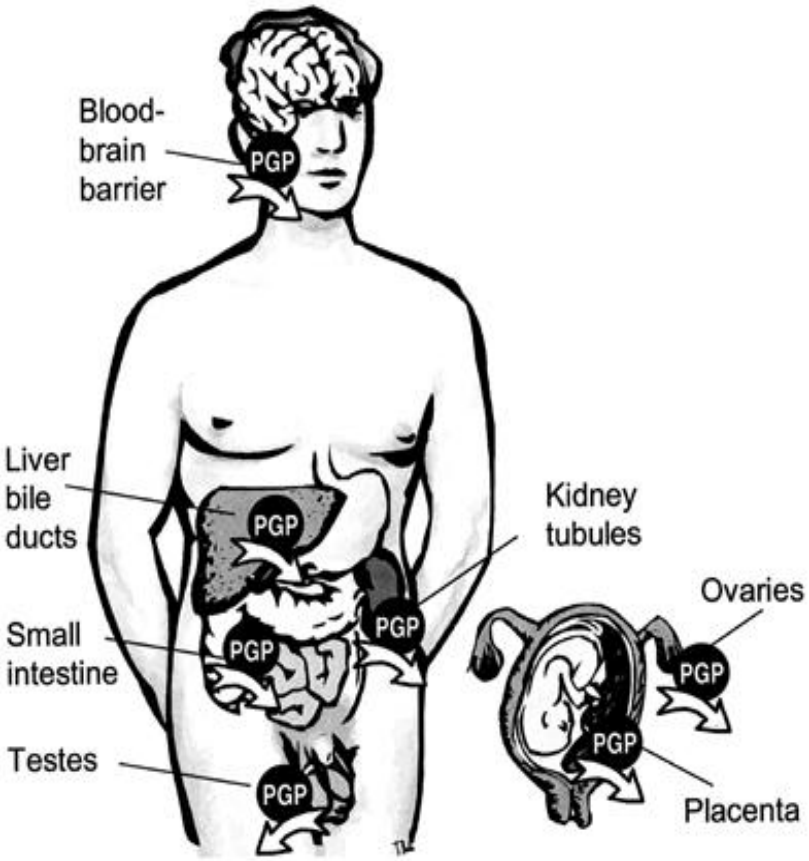


Figure 1.9: Distribution of P-gp within the body (Marzolini *et al.*, 2004)

1.3.1. Structure and function of P-glycoprotein

P-gp is encoded by the *ABCB1* (also known as *MDR1*) gene, located on chromosome 7 at q21 with 28 exons encoding a protein of 1,280 amino acids, in humans (Ieiri, 2012). It is a 170-kDa membrane-bound broad-spectrum efflux pump, consisting of twelve highly hydrophobic transmembrane domains, arranged as two bundles of six helices linked by a highly charged extracellular 'linker region', and two intracellular ATP-binding sites, known as nucleotide binding domains (NBDs) (Zhou, 2008; Aller *et al.*, 2009).

The hydrophobic 'vacuum cleaner' and flippase models have been proposed to describe P-gp-mediated drug translocation (Shapiro and Ling, 1997; Aller *et al.*, 2009; Colabufo *et al.*, 2010). In the hydrophobic 'vacuum cleaner' model, P-gp pulls its substrates from the lipid bilayer and pumps them out of the cell through its central cavity. According to the flippase model, P-gp 'scans' the inner leaflet of the lipid bilayer and binds specific lipids and hydrophobic drugs prior to their extrusion by 'flipping' the phospholipids from the inner to outer leaflets of the lipid bilayer. The flippase model and the hydrophobic 'vacuum cleaner' model are not mutually exclusive, and a combination of both models is illustrated in Figure 1.10 (Aller *et al.*, 2009; Eckford and Sharom, 2009). The active drug efflux process is powered by ATP hydrolysis at the cytoplasmic NBDs (Sharom, 2008).

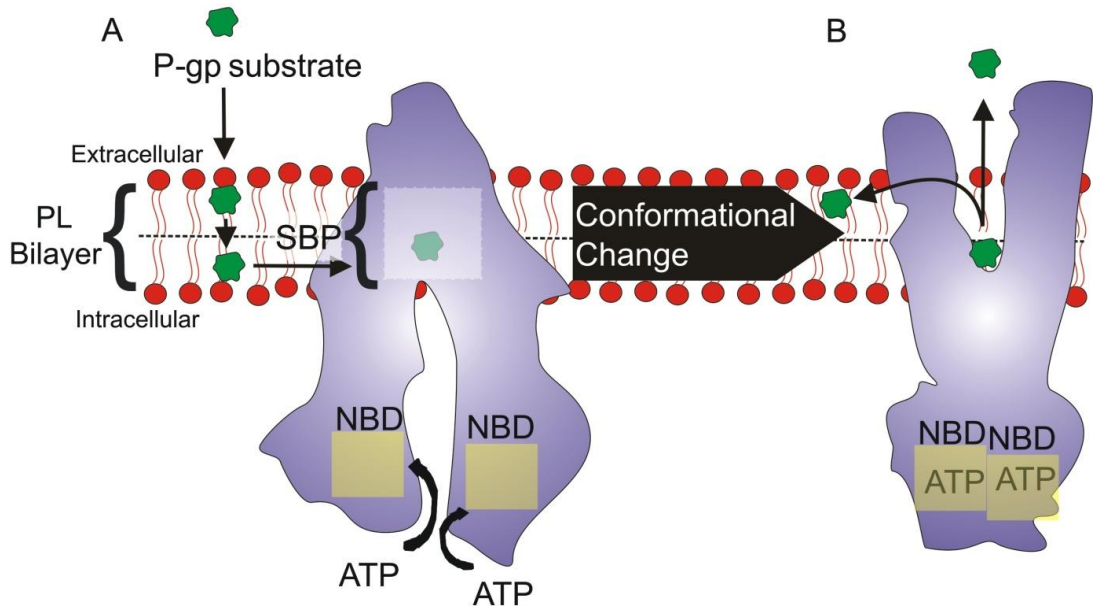


Figure 1.10: Schematic illustrating the mechanism of action of P-gp (O'Brien *et al.*, 2012b). *A. In its substrate-binding conformation, P-gp contains a large substrate-binding pocket (SBP), comprising mostly of hydrophobic and aromatic residues. Lipid soluble P-gp substrate molecules travel into the SBP from the inner leaflet of the phospholipid (PL) bilayer membrane. Substrate-P-gp interactions lead to the binding of two ATP molecules to the nucleotide binding domain (NBD). B. ATP-binding causes dimerization of the NBDs, leading to a conformational change, resulting in an outward facing configuration. This outward facing arrangement facilitates the release of substrates into the extracellular environment or the outer leaflet of the PL bilayer, and sterically prevents the substrate from travelling into the intracellular space. Thus, P-gp acts as a unidirectional efflux pump (see (Aller et al., 2009)).*

1.3.2. P-gp substrates and inhibitors

One of the hallmarks of P-gp is its substrate promiscuity, as it transports a wide range of structurally unrelated compounds (Table 1.4) (Aller *et al.*, 2009). Although P-gp substrates are generally hydrophobic amphipathic compounds, no chemical characteristic that clearly distinguishes between P-gp substrates and non-substrates has been determined (Schinkel, 1999). Interestingly, there is a significant overlap in substrate specificity between P-gp and drug metabolizing enzymes, such as cytochrome P450 (CYP) enzymes (Wacher *et al.*, 1995; Uhr *et al.*, 2004). Thus, P-gp and drug metabolizing enzymes in BCECs may work in tandem to reduce the ability of certain drug molecules, which are substrates of both, to pass through the BBB in a so-called "drug transporter-metabolism alliance" (as originally proposed in

relation to drug absorption from the gut) (Benet, 2009). According to this hypothesis, P-gp efflux would prevent the intact substrate from passing through the BBB, resulting in a cycle where drug molecules passively diffuse into the BCEC, followed by active P-gp mediated extrusion. Each time drug molecules diffuse into the BCEC in this cycle, a certain proportion of the molecules would be broken down by the drug metabolizing enzymes, while unchanged molecules would be recycled back out of the cell by P-gp.

Compounds that interact with P-gp can be classified as substrates, modulators or inhibitors (Colabufo *et al.*, 2010). Substrates are subjected to active efflux by P-gp, and therefore have a reduced ability to penetrate P-gp expressing membranes. Modulators reduce substrate binding via a negative allosteric interaction, thereby reducing P-gp-mediated substrate efflux; whereas inhibitors reduce P-gp function by interfering with the substrate or nucleotide binding steps (Colabufo *et al.*, 2010). Therefore, modulators and inhibitors achieve the same pharmacological effect, albeit via different mechanisms, and for the purposes of this thesis are considered synonymous. Some, but not all, P-gp substrates also reduce P-gp function, and as such can be classed as both substrates and inhibitors.

Table 1.4: List of selected P-gp substrates and inhibitors (O'Brien *et al.*, 2012b)

Drug class	Selected examples
P-gp substrates	
Anticancer agents	Daunorubicin, doxorubicin, etoposide, imatinib, methotrexate, mitoxantrone, paclitaxel, vinblastine, vincristine
Antidiarrhoeal agents	Loperamide
Antihistamines	Cetrizine, desloratadine, fexofenadine
Antipsychotics	Risperidone
Antiretroviral drugs	Amprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir
Cardioactive drugs	Amiodarone, digoxin, diltiazem, quinidine, verapamil
Antiemetics	Domperidone, ondansetron
β-blockers	Talinolol
H_2 receptor antagonists	Cimetidine, ranitidine
P-gp inhibitors	
First generation	Amiodarone, cyclosporin A, nifedipine, quinidine, verapamil
Second generation	Dexverapamil, GF120918 (elacridar), PSC-833 (valspodar), VX-710 (biricodar)
Third generation	LY-335979 (zosuquidar), LY475776, OC144-093, R-101933 (laniquidar), XR-9576 (tariquidar)

1.3.3. Regulation of P-gp expression and activity

In common with other ABC transporters, the expression and activity of P-gp at the BBB are altered in response to factors including xenobiotics, inflammation, infection and disease (Miller, 2010).

In rodents, the increase in P-gp expression and activity in response to xenobiotics is mediated via ligand-activated intracellular receptors, primarily the pregnane-X receptor (PXR) (known as the steroid and xenobiotic receptor (SXR) in humans (Loscher and Potschka, 2005b)) and the constitutive androstane receptor (CAR) (Bauer *et al.*, 2004; Bauer *et al.*, 2006; Wang *et al.*, 2010). Interestingly, as well as upregulating efflux transporters such as P-gp, activation of PXR and CAR also induces the expression of metabolic enzymes in a coordinated defensive response to xenobiotic exposure (Bauer *et al.*, 2008; Pascussi *et al.*, 2008; Kohle and Bock, 2009). Both receptors are activated by numerous endogenous ligands, including bile acids, and by several drugs, many of which are subjected to efflux or metabolism by the transporters and enzymes regulated by the receptors themselves (Miller, 2010). Furthermore, PXR and CAR have common activating ligands, including the clinically used drugs rifampicin, taxol, dexamethasone and phenobarbital (Kliewer *et al.*, 2002; Willson and Kliewer, 2002). In addition, PXR and CAR share common target genes, and both associate with the retinoid-X receptor before binding to DNA (Miller, 2010).

The importance of xenobiotic-mediated increases in efflux transporter expression were highlighted in a study in which transgenic mice expressing human PXR (hPXR) were treated with the hPXR ligand, and clinically used antibiotic, rifampicin (Bauer *et al.*, 2006). The dosing regimen, designed to achieve free plasma rifampicin levels equivalent to those achieved in patients undergoing course of rifampicin treatment, resulted in a 2-3 fold increase in P-gp protein levels in intestine, liver and brain capillaries. Moreover, this resulted in a 70% reduction in the antinociceptive effects of methadone, an analgesic drug which is a transported P-gp substrate (Bauer *et al.*, 2006). This demonstrates the potential pharmacodynamic consequences of xenobiotic-mediated drug transporter induction at the BBB.

In addition to xenobiotics, inflammation and immune activation can modulate P-gp expression and activity. These effects are highly complex and can result in up- or down-regulation of P-gp (Roberts and Goralski, 2008). In essence, acute inflammation or infection appears to result in reduced P-gp function and increased accumulation of P-gp substrates in the brain, whereas P-gp function is recovered or enhanced following chronic inflammation (Roberts and Goralski, 2008).

Furthermore, P-gp expression and activity may be altered in different disease states, particularly in neurodegenerative diseases, such as Alzheimer's disease for example (Vogelgesang *et al.*, 2002; Zlokovic, 2008). Indeed, β-amyloid (Aβ), a protein involved in the pathophysiology of Alzheimer's disease, is a transported substrate of P-gp *in vitro* and *in vivo* (Kuhnke *et al.*, 2007; Hartz *et al.*, 2010), while the deposition of Aβ is inversely correlated with P-gp expression in the brains of elderly non-demented humans (Vogelgesang *et al.*, 2002). Moreover, increasing P-gp expression by PXR activation results in reduced brain accumulation of Aβ in a mouse model of Alzheimer's disease (Hartz *et al.*, 2010). Thus, reduced P-gp function has been hypothesized to be involved in the pathophysiology of Alzheimer's disease, resulting in increased deposition of the pathological protein Aβ (Vogelgesang *et al.*, 2004; Zlokovic, 2008).

1.3.4. Single nucleotide polymorphisms in *ABCB1*

Genetic heterogeneity in *ABCB1* may influence P-gp functionality, and therefore lead to clinically relevant inter-individual differences in the pharmacokinetics of P-gp substrates. Indeed, more than 50 SNPs in ABCB1 have been identified in humans (Table 1.5) (Stein *et al.*, 1994; Hoffmeyer *et al.*, 2000; Cascorbi *et al.*, 2001; Ito *et al.*, 2001; Kim *et al.*, 2001; Cascorbi, 2006; Salama *et al.*, 2006; Kimchi-Sarfaty *et al.*, 2007; Sakurai *et al.*, 2007) (see also: http://pharmacogenetics.ucsf.edu/cgi-bin/Gene.py?hgncSymbol=*ABCB1*).

Most studies in this regard have focused on three SNPs in particular: rs1128503 (C1236T; exon 12), rs2032582 (T2677G; exon 21) and rs1045642 (C3435T; exon 26). However, despite an abundance of studies, the functional impact of these SNPs, or indeed of the others, remains largely unclear and somewhat controversial (Ieiri,

2012). Given the substantial influence of environmental factors, such as xenobiotics and infections (as discussed in Section 1.3.3), it is perhaps unsurprising that robust genetic effects have not been observed. Moreover, some of these SNPs exist in linkage disequilibrium with each other, and therefore focusing on individual SNPs, as opposed to their role in haplotypes, may have obscured potentially relevant genetic effects on P-gp functionality (Ambudkar *et al.*, 2003; Marzolini *et al.*, 2004).

Several studies have reported that specific *ABCB1* SNPs can have an impact on P-gp expression and function in humans, and therefore influence the pharmacokinetics of certain drugs which are transported substrates of P-gp (Hoffmeyer *et al.*, 2000; Johne *et al.*, 2002; Fukui *et al.*, 2007; Ieiri, 2012). The first such study reported that the synonymous C3435T SNP was associated with reduced P-gp expression in individuals homozygous for the T allele compared to those homozygous for the C allele (Hoffmeyer *et al.*, 2000). This resulted in higher steady-state plasma levels of the P-gp substrate digoxin following oral administration in those homozygous for the T allele, indicating that intestinal P-gp was not limiting its oral absorption to the same extent as in C/C carriers due to reduced P-gp expression (Hoffmeyer *et al.*, 2000). While this study has been replicated to an extent (Kurata *et al.*, 2002), others have reported directly contradictory findings, with lower digoxin bioavailability in T/T carriers (Sakaeda *et al.*, 2001). This example highlights inconsistencies in the available data in relation to the functional impact of *ABCB1* SNPs in general (Ieiri, 2012).

Table 1.5: List of selected *ABCB1* SNPs, associated amino acid changes and their frequencies in different ethnic groups

dbSNP ID	SNP	AA substitution	Mutant allele frequency (%)		
			African	Asian	Caucasian
rs9282564	A61G	Asn21Asp	2.5	1.7	8
rs2229109	G1199A	Ser400Asn	1	0	2.5
rs1128503	C1236T	Silent	20.9	68.5	45.9
rs2032582	G2677T	Ala893Ser	10	45	46.4
rs2032582	G2677A	Ala893Thr	0.5	6.7	3.6
rs1045642	C3435T	Silent	20.2	40	56.1
rs2235015	G→T	Intronic	30	6.7	15.8
rs2032583	T→C	Intronic	n/a	n/a	n/a
Mutant allele frequency data obtained from: http://pharmacogenetics.ucsf.edu					

1.3.5. The contribution of P-gp efflux to treatment failure

Efflux of P-gp substrate drugs has been linked to treatment failure in many diseases, most notably in cancer (Gottesman *et al.*, 2002; Szakacs *et al.*, 2006). Indeed, P-gp was first identified as a result of it conferring treatment resistance to certain anticancer agents *in vitro* (Dano, 1973; Juliano and Ling, 1976). Thus, the name 'multidrug resistance transporter' was originally coined for P-gp, and it is still often referred to by this term. Certain cancerous cells and tumours are known to overexpress P-gp (Gottesman *et al.*, 2002), and several cytotoxic drugs are avidly transported P-gp substrates (Table 1.4). As a result, such cytotoxic drugs can be ineffective in the treatment of P-gp overexpressing cancers, such as acute myeloid leukaemia (Leith *et al.*, 1999). Moreover, P-gp at the BBB contributes to the lack of efficacy of these anticancer agents in the treatment of brain tumours (Kemper *et al.*, 2004).

Much recent interest has focused on the role of P-gp in treatment resistance beyond oncology, with evidence implicating a potential involvement of P-gp efflux in drug-resistant brain diseases such as CNS HIV infection, epilepsy, schizophrenia and depression (Loscher and Potschka, 2005b).

Several antiretroviral drugs used in the treatment of HIV are transported substrates of P-gp (Table 1.4). In the CNS, HIV infection mostly targets microglia and, to a lesser extent, astrocytes (Speth *et al.*, 2005), and P-gp expression is upregulated in HIV-infected astroglial and microglial cells (Langford *et al.*, 2004). Therefore, P-gp efflux presents an obstacle to successful treatment of CNS HIV at two stages: firstly in limiting the distribution of antiretroviral drugs across the BBB, and secondly in preventing their intracellular accumulation within infected cells.

Approximately one third of patients with epilepsy are resistant to treatment (Kwan and Brodie, 2000). Multidrug resistance transporters, including P-gp, are overexpressed in BCECs and astrocytes in epileptogenic brain tissue from patients with refractory epilepsy (Tishler *et al.*, 1995; Dombrowski *et al.*, 2001; Aronica *et al.*, 2003; Aronica *et al.*, 2004; Feldmann *et al.*, 2013). Increasing *in vitro* and *in vivo* evidence indicates that many, but not all, commonly prescribed anti-epileptic drugs are transported substrates of efflux transporters, such as P-gp, at the BBB (Zhang *et al.*, 2012). Thus, overexpression of P-gp may prevent therapeutically effective concentrations of the antiepileptic drugs from reaching the epileptic neurons, thereby contributing to the high incidence of medically intractable epilepsy (Potschka, 2012; Zhang *et al.*, 2012). Indeed, preclinical and small-scale clinical studies investigating the effect of P-gp inhibition as an adjunctive strategy in epilepsy have reported promising results (Summers *et al.*, 2004; van Vliet *et al.*, 2006)

In the field of psychiatry, much recent interest has focused on the role of P-gp in the treatment of schizophrenia and depression (Loscher and Potschka, 2005b). Over 20% of patients with schizophrenia do not respond to treatment with antipsychotics (Van Sant and Buckley, 2011). The majority of clinically used antipsychotic drugs, such as amisulpride, aripiprazole risperidone and olanzapine, are transported substrates of P-gp at the BBB (El Ela *et al.*, 2004; Wang *et al.*, 2004a; Wang *et al.*, 2004c; Doran *et al.*, 2005; Wang *et al.*, 2009). Clozapine, on the other hand, is considered not to be a P-gp substrate (Schinkel *et al.*, 1996; El Ela *et al.*, 2004) and is generally accepted to be more effective than other antipsychotics

in the management of treatment-resistant schizophrenia (Kane *et al.*, 1988; Van Sant and Buckley, 2011), leading to speculation that its lack of transport by P-gp may contribute to its relative efficacy (Loscher and Potschka, 2005b). However, the evidence supporting a substantial role for P-gp in treatment resistant schizophrenia is not fully convincing, and further research is required before drawing conclusions in this regard, not least considering a more recent study reported that clozapine is a P-gp substrate (Doran *et al.*, 2005).

A growing body of preclinical and clinical evidence points toward a role for P-gp in antidepressant treatment. This topic is discussed in detail in Section 1.5.

1.3.6. P-gp inhibition as a strategy to augment the therapeutic effects of P-gp substrate drugs

Given the contribution of P-gp efflux to treatment failure, it has been proposed that co-administration of P-gp inhibitors with P-gp substrate drugs may represent a potential strategy to overcome P-gp-mediated drug resistance. Indeed, preclinical studies, both *in vitro* and *in vivo*, have offered hope that this strategy could prove to be beneficial in cancer and epilepsy (Fellner *et al.*, 2002; Clinckers *et al.*, 2005). In addition, small scale studies in humans have yielded promising preliminary results (List *et al.*, 2001; Summers *et al.*, 2004).

However, larger scale clinical trials to date, which have focused primarily on the use of P-gp inhibitors in combination with cytotoxic drugs in treatment resistant cancer, have not proven to be successful due to pharmacokinetic and pharmacodynamic limitations, largely associated with the toxicity of the chemotherapeutic drug or a lack of therapeutic benefit (Szakacs *et al.*, 2006; Colabufo *et al.*, 2010; Shaffer *et al.*, 2012).

For example, a high profile failure in this field involved a Phase III clinical trial of the third generation P-gp inhibitor tariquidar as an adjunctive therapy in non-small cell lung cancer (NSCLC) (http://clinicaltrials.gov/show/NCT00042302). This trial, involving co-administration of the tariquidar and the cytotoxic drugs paclitaxel and carboplatin in patients with advanced stage NSCLC, was terminated prematurely

due to safety concerns. Tariquidar administration exacerbated side-effects associated with the cytotoxic drugs, without improving patient outcomes (Szakacs *et al.*, 2006). The failure of this study was perceived as a major blow in the development of P-gp inhibitors as a clinical augmentation strategy, despite the fact that there were fundamental flaws in the study design (Szakacs *et al.*, 2006). Firstly, NSCLC has not been associated with P-gp overexpression, meaning that there was no clear rationale underpinning adjunctive treatment with a P-gp inhibitor (Szakacs *et al.*, 2006). Secondly, the dose of tariquidar used in the clinical trial has been subsequently revealed to be inadequate to achieve substantial inhibition of P-gp (Kreisl *et al.*, 2010).

Despite these setbacks, the availability of specific and potent modulators of P-gp function at the BBB may lead to a therapeutically useful role for P-gp inhibitors in the future. In particular, the potential of P-gp inhibition as an adjunctive strategy in treatment resistant diseases aside from cancer, thereby avoiding problems associated with potentiation of cytotoxic activity in healthy tissues, remains underinvestigated. It should be noted, however, that a recent paper, published on behalf of the International Transporter Consortium, questions whether clinical modulation of efflux transport at the human BBB is likely to become a therapeutic option in future (Kalvass *et al.*, 2013).

1.3.7. Methods to assess drug interactions with P-gp at the BBB

Several different methodological approaches, ranging from *in silico* to *in vivo* and human imaging studies, can be employed to assess drug interactions with P-gp. Such studies can focus on drug-P-gp interactions in general, or with a specific emphasis on interactions at the BBB in particular.

1.3.7.1. In silico approaches

As outlined in Section 1.2.5.1, attempts to predict or identify transported substrates of efflux transporters based on structural properties have proven to be challenging (Sharom, 2008). Recent advancements have led to more complex algorithms and a better understanding of molecular characteristics that increase the likelihood of being subjected to transport by P-glycoprotein (Desai *et al.*, 2013). For example,

most transported substrates of P-gp possess one or two hydrophobic centres, an aromatic ring, electron donor and/or hydrogen bond donor and acceptor groups (Raub, 2006). This knowledge can prove useful in the optimisation and selection of lead compounds for further investigation, based on a desire to select substrates or non-substrates of P-gp. However, *in vitro* or *in vivo* experimental approaches remain the only reliable method to confidently identify transported substrates of P-gp.

1.3.7.2. In vitro studies

Several *in vitro* screening assays can be used to determine interactions between test compounds and P-gp, and thereby classify drugs as P-gp substrates or inhibitors (Polli *et al.*, 2001). Functional assays used to identify P-gp substrates include the ATPase activity and bidirectional transcellular transport assays.

The relationship between P-gp activity and the breakdown of ATP is exploited in the ATPase activity assay, whereby the release of inorganic phosphate as a result of ATP hydrolysis following drug incubation with a P-gp expressing membrane is monitored (Sarkadi *et al.*, 1992). This facilitates high-throughput identification of compounds that interact with P-gp. However, many compounds identified as so-called P-gp 'substrates' using this assay may not be subject to significant P-gp transport (Schwab *et al.*, 2003), and this is a major limitation of the ATPase activity assay. As a result, this assay is of limited utility in the accurate identification of compounds that are meaningfully subjected to P-gp-mediated transport (Balimane *et al.*, 2006).

Bidirectional transcellular transport assays represent the gold standard for identifying P-gp substrates *in vitro*, and are the most accurate predictive model for the identification of P-gp substrates *in vivo* (Balimane *et al.*, 2006; Feng *et al.*, 2008) (Figure 1.11). However, these monolayer efflux assays are labour intensive and, consequently, time consuming with a relatively low-throughput.

Many cell-lines suitable for use in bidirectional transcellular transport assays are available, including the naturally P-gp-expressing human colorectal adenocarcinoma (Caco-2) cells and *ABCB1*-transfected Madine-Darby canine kidney

cells (MDCK-MDR1). These model cell-lines are polarized, with distinct apical and basolateral membrane domains, when cultured on an appropriate transwell support. As P-gp is exclusively expressed on the apical membrane of the cultured cells, the comparison of apical-to-basolateral (A→B) permeability and basolateral-to-apical (B→A) permeability for a test compound indicates if it is a P-gp substrate. A (B→A)/(A→B) transport ratio (TR) of > 2.0 is indicative of P-gp mediated efflux (Lin, 2007).

The presence of other endogenous efflux transporters, in addition to P-gp, in these cell systems may influence transport. Therefore, it is advisable to use specific and potent P-gp inhibitors, in conjunction with the TR, to determine if P-gp mediated efflux is the transport mechanism at play, and thus if the test compound is a P-gp substrate. Furthermore, for *ABCB1*-transfected cell lines, such as MDCK-MDR1, a 'corrected' transport ratio (cTR) can be calculated by dividing the TR obtained in the *ABCB1*-transfected cells by the TR obtained in the respective wild-type cell line (Zhang *et al.*, 2006; Kuteykin-Teplyakov *et al.*, 2010). However, the expression of endogenous drug transporters in these cell lines can be affected by transfection with the human *ABCB1* gene (Kuteykin-Teplyakov *et al.*, 2010). This must be taken into consideration when interpreting cTR values.

Similarly, known P-gp substrates can be used in the bidirectional transcellular transport assay to determine if a test compound is a P-gp inhibitor. P-gp substrates commonly used for this purpose include digoxin, loperamide and talinolol (Pariante *et al.*, 2003b; El Ela *et al.*, 2004; Zhang *et al.*, 2006). A decrease in the TR for the P-gp substrate following co-incubation with the test compound is indicative of P-gp inhibition by the test compound.

Other assays, which are less laborious and therefore offer higher throughput, can be used for the identification and classification of P-gp inhibitors. These P-gp inhibition assays utilise fluorescent P-gp substrates, such as calcein acetoxymethyl ester (calcein AM) or rhodamine 123, can be easily automated, and have the considerable advantage of a generic fluorescent output. Increased intracellular accumulation (as measured by increased fluorescence) of the fluorescent P-gp

substrate in P-gp expressing cells following co-incubation with the test compound thus gives an indirect measure of P-gp inhibition (Varga *et al.*, 1996; Ibrahim *et al.*, 2000; Stormer *et al.*, 2001; Weiss *et al.*, 2003; Feng *et al.*, 2008). Similarly, the susceptibility of P-gp-expressing-cells to P-gp-substrate cytotoxic drugs, such as doxorubicin or vinblastine, can be determined. Enhanced cytotoxicity in the presence of the test compound is indicative of P-gp-inhibition (Merry *et al.*, 1991; Jaffrezou *et al.*, 1995).

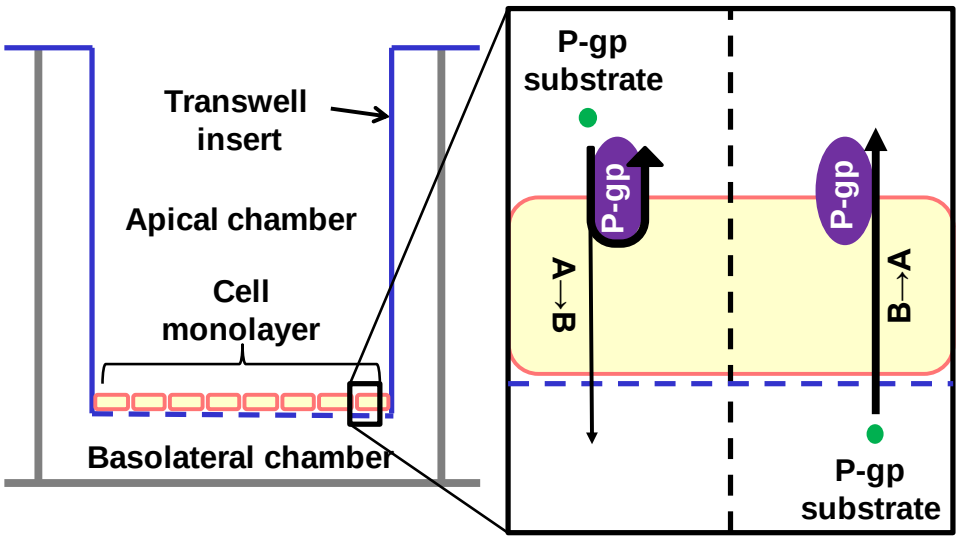


Figure 1.11: Schematic of *in vitro* bidirectional transport assay. *A cell line expressing P-gp in a polarised fashion, on the apical membrane only (e.g. MDCK-MDR1), is cultured as a monolayer on a semipermeable membrane at the base of a transwell insert. Tight junctions form between the cells, thus limiting paracellular transport. Transport studies are carried out in both the apical-to-basolateral (A→B) direction, by adding the compound of interest to the apical chamber and sampling from the basolateral chamber, and the basolateral-to-apical (B→A) direction. Based on these experiments, the permeability of the compound in each direction can be calculated, and the permeability in the B→A direction can be compared to the A→B direction to yield a transport ratio. As P-gp, expressed on the apical membrane only, will limit the permeability of its transported substrates in the A→B direction, but not in the B→A direction, P-gp substrates will exhibit greater permeability in the B→A direction than the A→B direction, thus yielding a transport ratio greater than 1. Non-substrates of P-gp will yield transport ratios of approximately 1.*

1.3.7.3. *In vivo studies*

While P-gp is encoded by a single gene in humans (*ABCB1*), its functions are performed by two homologues in mice: the *abcb1a* and *abcb1b* genes (Devault and Gros, 1990). The overall tissue distribution of P-gp overlaps well between the two species (Ebinger and Uhr, 2006), and there seems to be a high level of correlation in substrate specificities between mouse and human P-gp based on *in vitro* studies (Feng *et al.*, 2008), despite concern relating to potential species differences (Yamazaki *et al.*, 2001; Katoh *et al.*, 2006; Baltes *et al.*, 2007; Syvanen *et al.*, 2009). Thus, it appears that mouse-based models are useful, if imperfect, tools for predicting P-gp-drug interactions in humans.

Abcb1a P-gp is highly expressed at the murine BBB, while it appears that *abcb1b* P-gp is also expressed at the BBB, but to a much lesser extent (Pariante, 2008). Thus the generation of *abcb1* knockout mice, firstly single knockout *abcb1a*$^{(-/-)}$ mice in 1994 (Schinkel *et al.*, 1994), followed by double knockout *abcb1ab*$^{(-/-)}$ in 1997 (Schinkel *et al.*, 1997), has facilitated the study of the influence of P-gp on brain pharmacokinetics of various drugs. P-gp deficient mice display normal viability, thereby indicating that organisms can survive with a complete absence of P-gp activity (Schinkel *et al.*, 1997). This is an important consideration when evaluating P-gp inhibition as a potential treatment strategy in patients.

By comparing brain/plasma drug concentration ratios between these P-gp-deficient knockout mice to those of wild-type controls, it has been possible to identify drugs as P-gp substrates *in vivo*. Alternatively, established P-gp inhibitors, such as verapamil and cyclosporin A, can be administered to wild-type animals to investigate the influence of P-gp inhibition on brain pharmacokinetics.

1.3.7.4. *Human studies*

Positron emission tomography (PET) imaging can be used to measure P-gp function in human volunteers or patient populations, using radiolabelled substrates, such as $^{99m/94m}$Tc-sestamibi, ^{11}C-verapamil, ^{18}F-paclitaxel or ^{11}C-*N-desmethyl*-loperamide (de Klerk *et al.*, 2009; Wagner *et al.*, 2009; Kreisl *et al.*, 2010). Assessing P-gp function in this way could be useful in predicting drug resistance in patients to be treated with

P-gp substrate therapeutics, identifying disease-states which are associated with alterations in P-gp activity or in assessing the ability of compounds to inhibit P-gp at different doses in humans (de Klerk *et al.*, 2009; Wagner *et al.*, 2009; Kreisl *et al.*, 2010). However, imaging techniques are not generally used to identify drugs which are subject to P-gp-mediated efflux at the BBB, as it would be necessary to generate radio-labelled versions of each drug of interest to carry out such assessments.

1.4. Depression

Clinical depression is a common and devastating mood disorder, affecting more than 350 million people globally (World Health Organisation, 2012). According to the World Health Organisation, depression is the leading cause of disability worldwide, and is a major contributor to the global burden of disease (World Health Organisation, 2012).

Major depressive disorder is diagnosed based on symptomatic criteria, such as those outlined in the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5; Table 1.6) (American Psychiatric Association, 2013) or the *International Classification of Diseases* (ICD). Suicide is an all-too-common tragic consequence of depression; alarmingly suicide was the tenth leading cause of death in the United States in 2010 (Centers for Disease Control and Prevention, 2013), and is a well-recognised problem in Ireland and beyond. Given the severity of the symptoms associated with depression and its widespread prevalence, there is a major need to unravel the pathophysiology of the disorder, and to achieve optimal treatment strategies.

Table 1.6: Symptoms of major depressive disorder (DSM-5)

	Symptom
1	Depressed mood or irritable most of the day, nearly every day
2	Decreased interest or pleasure in most activities, most of each day
3	Significant weight change (5%) or change in appetite
4	Change in sleep: either insomnia or hypersomnia
5	Change in activity: psychomotor agitation or retardation
6	Fatigue or loss or energy
7	Feelings of worthlessness or excessive/inappropriate guilt
8	Diminished ability to think or concentrate, or increased indecisiveness
9	Thoughts of death or suicide, or having a suicide plan
N.B.	At least 5 of these 9 symptoms, including one of the first two, present nearly every day for more than two weeks for clinical diagnosis of major depressive disorder

1.4.1. Neurobiology of depression

While considerable advances have been made since Hippocrates proposed that an excess of black bile was the cause of *melancholia*, the neurobiological mechanisms underlying depression, and its exact aetiology, remain unclear despite extensive research (Cryan and Holmes, 2005; Krishnan and Nestler, 2008). Genetic and environmental factors, such as stress in particular, play an important role in conferring susceptibility to depression, with gene-environment interactions thought to be particularly important (Wong and Licinio, 2001). Numerous hypotheses, involving monoaminergic, neurotrophic, neuroendocrine and neuroimmune mechanisms, have been proposed to underpin the neurobiological changes that result in depression (Wong and Licinio, 2001; Duman and Monteggia, 2006; Krishnan and Nestler, 2008; Leonard and Myint, 2009). While each of these hypotheses has merit, none offers a comprehensive explanation of depression on its own, as several mechanisms are likely to contribute to what is a symptomatically, biologically and genetically heterogeneous disorder (Krishnan and Nestler, 2008).

Over 50 years ago, serendipitous observations in relation to reserpine and iproniazid and imipramine, drugs developed as antihypertensive, antituberculosis and antihistamine agents, respectively, led to the 'monoamine hypothesis' of

depression, which postulates that depression occurs as a result of reduced monoaminergic transmission in the brain (Slattery *et al.*, 2004; Krishnan and Nestler, 2008). Reserpine treatment resulted in a depletion of monoamine stores, inducing depressive symptomatology in a subset of patients, whereas treatment with iproniazid or imipramine, which were later shown to enhance central serotonin and noradrenaline neurotransmission, was found to elevate mood in depressed patients. However, the cause of depression is far from being a simple case of deficiencies in monoaminergic signalling, as highlighted by limitations of the monoamine hypothesis of depression (Krishnan and Nestler, 2008). While monoamine-based antidepressant drugs elicit rapid increases in monoamine transmission, it requires weeks of treatment for them to produce a clinically relevant enhancement of mood. Moreover, a high proportion of depressed patients do not respond to treatment with these antidepressants at all (Fava, 2003). Thus, it is now thought that acute increases in monoamine transmission by antidepressants result in secondary neuroplastic changes which require a longer timescale to exert their effect on mood (Krishnan and Nestler, 2008). Nonetheless, the vast majority of clinically used antidepressants today act to increase monoamine neurotransmission acutely, as efforts to identify and exploit other druggable targets in depression have mostly proven to be unsuccessful (Table 1.7).

Another popular hypothesis pertaining to the pathogenesis of depression involves neurotrophins and neurogenesis (Duman and Monteggia, 2006). Stress, one of the main precipitating factors of depression in humans (Gold and Chrousos, 2002), and antidepressant treatment have opposing effects on neurotrophic factors (Duman and Monteggia, 2006). Preclinical studies have revealed that stress decreases the expression of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), in limbic structures associated with mood, and that these effects are reversed or blocked by antidepressant treatment (Duman and Monteggia, 2006). Similar BDNF deficits have been observed in post-mortem hippocampus samples from suicide victims with depression (Karege *et al.*, 2005). Moreover, neuroimaging and post-mortem studies have revealed volumetric deficits in specific brain regions, including the prefrontal cortex and hippocampus, in subsets of depressed patients

(Cotter *et al.*, 2001; Sheline, 2003), while preclinical studies have demonstrated that several antidepressants induce adult hippocampal neurogenesis (Sahay and Hen, 2007). Taken together, these observations support the concept that deficits in neurotrophins, such as BDNF, may be at play in depression. Indeed, much preclinical evidence points to a role for BDNF in depression and antidepressant response (Duman and Monteggia, 2006). Moreover, direct infusion of BDNF into the hippocampus induces an antidepressant-like effect in rodent models of depression, such as the forced swim test or the learned helplessness model (discussed in more detail in Section 1.4.5) (Siuciak *et al.*, 1997; Shirayama *et al.*, 2002; Hoshaw *et al.*, 2005). However, the 'BDNF hypothesis of depression' has drawn criticism, as further preclinical studies failed to replicate some of these findings, with some reporting directly opposing effects (Groves, 2007). Thus, it now appears that, while BDNF-mediated signalling is involved in neuroplastic responses to stress and chronic antidepressant treatment, these effects are both region- and antidepressant-specific (Krishnan and Nestler, 2008).

Small increases in serum glucocorticoid levels are commonly reported in depressed patients (Holsboer, 2000; Parker *et al.*, 2003; Julio-Pieper and Dinan, 2010), pointing to a potential role for dysregulation of the stress response via the hypothalamic-pituitary-adrenal axis in the pathophysiology of depression. This hypothesis is supported by the fact that some depression-like symptoms can by induced in rodents by chronic administration of glucocorticoids (Gourley *et al.*, 2008), while depression is common among patients with Cushing's syndrome, a feature of which is extreme hypercortisolaemia (McEwen, 2007). Moreover, excess glucocorticoids can reduce proliferation rates in the hippocampus, thereby potentially contributing the volumetric deficits in hippocampal volume observed in depression (a trait also seen in Cushing's syndrome), as discussed above (McEwen, 2007).

Further evidence points to a potential involvement of neuroimmune mechanisms in depression, resulting in the 'cytokine or inflammatory hypothesis' of depression (Raison *et al.*, 2006; Leonard, 2010). Depression is a common side-effect of

cytokine-treatment with interferons or interleukins for diseases such as hepatitis C, multiple sclerosis and cancer, affecting over 30% of patients (Loftis and Hauser, 2004; Dantzer *et al.*, 2008; Leonard, 2010), and elevated levels of the cytokine IL-6 have been observed in depressed patients (Maes *et al.*, 1995). Furthermore, depressive disorders occur more frequently in diseases involving immune dysfunction; cytokines can activate the HPA axis (a common trait in depression, as discussed above); and cytokines can influence monoaminergic signalling in the brain (which has been associated with depression as per the 'monoamine hypothesis') (Dunn *et al.*, 2005). Taken together, these associations indicate that immune activation may play a role in at least a subset of depression cases.

Relatively recent findings have triggered an interest in the glutamate system in depression (Cryan and O'Leary, 2010). Sub-anaesthetic doses of ketamine, a psychomimetic non-competitive antagonist of the n-methyl-d-aspartate (NMDA) glutamate receptor, have been reproducibly shown to elicit a rapidly acting, but transient, antidepressant effect in patients who have not responded to other antidepressant treatments (Berman *et al.*, 2000; Zarate *et al.*, 2006). However, while the NMDA receptor, and downstream targets such as mTOR (Li *et al.*, 2010), represent promising novel targets for drug treatment, at present there is limited evidence to suggest an important role for glutamatergic dysfunction in depression *per se* and further studies are required to more fully characterize the potential mechanisms at play (Krishnan and Nestler, 2008).

1.4.2. Treatment of depression

As stated earlier in Section 1.4.1, serendipity has played a bigger role than hypothesis-driven drug discovery in the development of treatments for depression to date (Slattery *et al.*, 2004).

Both pharmacological and non-pharmacological treatment strategies for the treatment of depression exist (Table 1.7). In general, more invasive non-pharmacological strategies, such as electroconvulsive therapy (ECT) or deep brain stimulation, are reserved for severe cases in which other approaches have failed (Hollon *et al.*, 2002).

Antidepressant drugs are categorised into different classes, based on structural characteristics and/or mechanisms of action, including selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) (Hollon *et al.*, 2002; Joint Formulary Committee, 2013). However, with the possible exception of agomelatine, a recently approved melatonin receptor agonist and 5-HT_{2C} receptor antagonist, all currently available antidepressant drugs, regardless of class, primarily act by enhancing monoaminergic transmission (Table 1.7). Substantial effort has been invested in attempts to identify, and exploit, novel therapeutic targets in depression. However, these efforts have generally not yet translated into alternative therapeutic options (Berton and Nestler, 2006).

There is little to choose between different antidepressants in terms of efficacy, although there is a marked difference in terms of safety and side-effects (Hollon *et al.*, 2002). Therefore, selection of an antidepressant for a particular patient is based on personal and familial history, the likelihood of particular side effects, safety in overdose and expense (Hollon *et al.*, 2002). SSRIs are considered first-line, as they are better tolerated and safer in overdose than other classes. TCAs, for example, are more likely to be discontinued due to side-effects, and, moreover, can be fatally toxic in cases of overdose. This is an important consideration, given that patients suffering from depression are at a higher risk of suicide. MAOIs, on the other hand, have dangerous interactions with some foods and drugs (refer to Section 1.4.3), and they are reserved for use by specialists (Hollon *et al.*, 2002; Joint Formulary Committee, 2013).

Table 1.7: List of currently available antidepressant treatments (adapted from Berton and Nestler, 2006; Kupfer *et al.*, 2012; Joint Formulary Committee, 2013)

Antidepressant class	Drugs (subclass)	Proposed mechanism of action
Pharmacological treatments		
TCAs	Amitriptyline, clomipramine, dosulepin, doxepin, Imipramine, lofepramine, nortriptyline, trimipramine	Enhance monoaminergic transmission by non-selectively inhibiting the reuptake of serotonin, dopamine and noradrenaline
TCA-related	Mianserin (NaSSA), trazodone	Modulation of monoaminergic system
MAOIs	**Irreversible:** phenelzine, isocarboxazid, tranylcypromine; **Reversible:** moclobemide	Enhance monoaminergic transmission by preventing the breakdown of monoamines by inhibiting the monoamine oxidase enzyme
SSRIs	Citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine, sertraline	Enhance monoaminergic transmission by selectively preventing the reuptake of serotonin
Other	Agomelatine	Melatonergic agonist (MT1 and MT2 receptors) and 5-HT_{2C} antagonist.
	Duloxetine, Venlafaxine (SNRIs)	Enhance monoaminergic transmission by preventing the reuptake of serotonin and noradrenaline
	Flupentixol	Dopamine receptor blockade (primarily an antipsychotic)
	Mirtazapine (NaSSA)	Increases central noradrenergic and serotonergic neurotransmission by inhibition of α_2 and 5-HT_{2C} presynaptic autoreceptors.
	Reboxetine (NRI)	Enhances noradrenergic transmission by preventing its reuptake
	Tryptophan (serotonin precursor)	Enhances serotonergic transmission by increasing central serotonin levels

Antidepressant class	Drugs (subclass)	Proposed mechanism of action
Non-pharmacological treatments		
	Electroconvulsive therapy (ECT)	General brain stimulation
	Magnetic stimulation	General brain stimulation? A magnetic field is thought to affect the brain by inducing electric currents and neuronal depolarization
	Vagal nerve stimulation (VNS)	Unknown
	Psychotherapy	Exact mechanism is uncertain, but is thought to involve learning new ways of coping with problems
	Deep brain stimulation	In severely ill patients, stimulation of a region of the cingulate cortex found to function abnormally in brain imaging scans reportedly has antidepressant effects
MAOIs: monoamine oxidase inhibitors; NaSSA: noradrenergic and specific serotonergic antidepressant; NRI: noradrenaline reuptake inhibitor; SNRIs: serotonin-noradrenaline reuptake inhibitors; SSRI: selective serotonin reuptake inhibitors; TCAs: tricyclic antidepressants		

1.4.3. Side-effects of antidepressant drugs

While all antidepressants are associated with side-effects, both central and non-central, some are better tolerated than others. The side-effect profiles of antidepressants are an important clinical consideration, and can be the primary discriminating criterion when it comes to antidepressant choice. Further, antidepressant side effects can be an important factor contributing to poor adherence to treatment regimens among patients and, therefore, treatment failure (Hollon *et al.*, 2002; Stahl, 2013).

As mentioned in Section 1.4.2, TCAs are associated with more severe side-effects than SSRIs, and are resultantly more likely to be discontinued due to adverse reactions, with on average 30% of patients ceasing treatment for this reason (Hollon *et al.*, 2002). Cardiovascular side-effects, such as arrhythmias, are a particular concern with TCAs, especially amitriptyline, and may be a contributory factor in sudden death of patients with underlying cardiovascular diseases. TCAs are associated with many other troublesome side-effects due to their off-target effects: blockade of H1 histamine receptors causes sedation and weight gain; anticholinergic actions at muscarinic cholinergic receptors cause dry mouth, urinary retention, constipation and memory disturbance; and orthostatic hypotension can occur due to blockade of α1 adrenoceptors in the brain (Hollon *et al.*, 2002; Stahl, 2013). Primarily due to these problematic side-effects, and the potentially fatal consequence of overdose, TCAs are no longer considered first-line options in the treatment of depression.

MAOIs, on the other hand, have potentially serious interactions with a wide variety of other medications and foods. As a result, they have not been prescribed widely since the 1960s, despite their clinical efficacy, and it is advised that they should only be prescribed by specialists (Hollon *et al.*, 2002). Ingestion of tyramine rich foods, such as mature cheese, while taking an MAOI may result in a dangerous increase in blood pressure as a result of a massive noradrenaline release, with potentially lethal consequences (Hollon *et al.*, 2002; Stahl, 2013). Furthermore, concurrent, or indeed consecutive if an insufficient wash-out period is allowed, treatment with

MAOIs and other antidepressants may precipitate the potentially fatal serotonin syndrome (see Section 1.4.3.1).

In comparison to TCAs and MAOIs, SSRI antidepressants are much better tolerated (Hollon *et al.*, 2002). Nevertheless, SSRI treatment is associated with many side effects, mainly as a result of increased serotonergic signalling: gastrointestinal side-effects, such as cramps and diarrhoea, via peripheral 5-HT_3 and 5-HT_4 receptors in the gut; centrally-mediated side-effects; including anxiety, appetite dysregulation, insomnia, sexual dysfunction, nausea and vomiting (Hollon *et al.*, 2002; Stahl, 2013). While the side effects of SSRIs are generally less problematic and less dangerous than those associated with other classes of antidepressants, they nonetheless represent a significant clinical problem and can result in poor patient adherence, with discontinuation of treatment in 10-20% of patients (Hollon *et al.*, 2002). Indeed, recent regulatory guidance recommended that doses of the commonly prescribed SSRIs citalopram and escitalopram should be minimized due to cardiovascular side-effects involving prolongation of the QT interval (Medicines and Healthcare products Regulatory Agency, 2011). Thus, any potential strategy to increase the selective delivery of antidepressants to their site of action in the brain, minimizing peripheral exposure and thereby limiting peripheral side effects, could be of enormous clinical benefit. However, enhancing brain concentrations of an antidepressant drug could result in an increased risk of centrally-mediated side-effects, such as those listed above.

1.4.3.1. Serotonin syndrome

Serotonin syndrome is a potentially fatal adverse drug reaction, which occurs as a result of excessive serotonin signalling following ingestion of agents which enhance serotonergic neurotransmission (Boyer and Shannon, 2005). It is characterised by clinical symptoms such as tremor, clonus, muscular hypertonicity, altered mental state and hyperthermia in patients (Boyer and Shannon, 2005). Examples of agents which may precipitate serotonin syndrome are listed in Table 1.8 (Boyer and Shannon, 2005; Haberzettl *et al.*, 2013). While serotonin syndrome may occur after ingestion of a single 5-HT enhancing agent, it is more commonly associated with

intake of two or more serotonergic drugs with differing mechanisms of action, such as SSRIs and MAOIs (Haberzettl *et al.*, 2013). Indeed, life-threatening cases are generally only observed where a combination of agents have been taken (Gillman, 2005).

Table 1.8: List of selected compounds known to contribute to the development of serotonin syndrome (adapted from Boyer and Shannon, 2005; Thanacoody, 2012; Haberzettl *et al.*, 2013)

Classification	Selected examples
SSRI antidepressants	Citalopram, escitalopram, fluoxetine, fluvoxamine, paroxetine, sertraline
MAOI antidepressants	Clorgiline, moclobemide, phenelzine, isocarboxazid
Other antidepressants	Buspirone, clomipramine, nefazodone, trazodone, venlafaxine
Anticonvulsants	Valproate
Analgesics	Fentanyl, pentazocine, pethidine, tramadol
Anti-emetic agents	Granisetron, ondansetron and metoclopramide,
Anti-migraine drugs	Sumatriptan
Bariatric medications	Sibutramine
Antibiotics/antiretrovirals	Linezolide (inhibits MAO) and ritonavir (inhibits CYP3A4)
Cough remedies	Dextromethorphan
Drugs of abuse	methylenedioxymethamphetamine (MDMA/'ecstasy'), lysergic acid diethylamide (LSD), cocaine
Dietary supplements and herbal products	Tryptophan, 5-hydroxy-tryptophan, *Hypericum perforatum* (St John's Wort)
Mood-stabilizers	Lithium

Owing to a lack of comprehensive epidemiological data pertaining to serotonin syndrome, it is not possible to definitively state its prevalence. However, estimates have approximated an incidence of 0.4 cases per 1,000 patient-months of treatment (Mackay *et al.*, 1999). Moreover, the incidence of serotonin syndrome is thought to be increasing as a result of better recognition by clinicians and greater prescribing of serotonergic drugs in recent years (Thanacoody, 2012). Given its

serious and potentially lethal consequences, it is ethically unacceptable to conduct human experiments to investigate drug combinations which may induce serotonin syndrome.

In this context, animal models of serotonin syndrome are an invaluable tool to investigate drug combinations which may precipitate the syndrome in humans (Haberzettl *et al.*, 2013). Many of the symptoms of serotonin syndrome can be successfully emulated in rodent models, which exhibit high levels of predictive validity (Haberzettl *et al.*, 2013). Behaviours associated with rodent models of serotonin syndrome include tremor, hind limb abduction and involuntary tics. These behaviours can be scored according to their severity, thereby facilitating quantitative evaluation of the impact of a drug on the manifestation of serotonin syndrome (Haberzettl *et al.*, 2013).

1.4.4. Treatment-resistant depression

Despite the numerous drugs available for the treatment of depression (Table 1.7), treatment resistant depression (TRD) represents a significant clinical challenge (Souery *et al.*, 2006). While the exact definition of TRD remains somewhat contentious, it is clear that current antidepressant therapies have unsatisfactory efficacy (Souery *et al.*, 2006; Holtzheimer and Mayberg, 2011). It has been estimated that up to 60% of depressed patients do not achieve an adequate response to antidepressant treatment (Fava, 2003; Trivedi *et al.*, 2006), with remission rates declining even further following successive treatment failures (Rush *et al.*, 2006). Moreover, relapse occurs in a large proportion of patients who initially respond to treatment (Holtzheimer and Mayberg, 2011). As depression is one of the leading causes of disability in the developed world (Cryan and Leonard, 2010), there is a major impetus to determine why so many patients fail to respond to antidepressant treatment, and to devise novel strategies to improve treatment outcomes.

Existing medication-based strategies recommended after the failure of an initial antidepressant trial, which has been optimized in terms of dose and duration, include augmentation with an adjunctive agent not thought to be an antidepressant

in its own right or switching to another antidepressant drug (Connolly and Thase, 2011). A multisite, prospective, randomized, multistep clinical trial of outpatients with nonpsychotic major depressive disorder, the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study, was undertaken in recent years to determine the effectiveness of existing treatment strategies for people with major depression who did not respond to initial treatment with the SSRI antidepressant citalopram (Rush *et al.*, 2004). This study found that, in the majority of patients, achieving remission of depressive episodes requires repeated trials of sufficiently sustained, vigorously dosed antidepressant therapy. Furthermore, switching antidepressant medication or augmenting it with another agent were found to be reasonable strategies in cases of initial treatment failure (Gaynes *et al.*, 2008).

Numerous agents, including lithium and thyroid hormone, have been proposed to augment the effects of antidepressants (Thase, 2004; Connolly and Thase, 2011). The modes of action of these strategies are not fully understood. Augmentation with lithium, primarily used as a mood stabiliser, is thought to enhance the effects of antidepressant treatment via modulation of second messenger systems or increases in hippocampal proliferation, while thyroid hormone may act by 'priming' noradrenergic neurotransmission or by correcting suboptimal thyroid function in a subset of depressed patients (Thase, 2004; O'Leary *et al.*, 2013). Although adjunctive lithium and thyroid hormone have established efficacy in the treatment of depression in particular circumstances, their relevance to modern practice is unclear. Most of the supporting evidence for these agents comes from trials when they were used in combination with TCAs in less severely resistant patients than those typically in receipt of TCAs nowadays (Connolly and Thase, 2011). As a result, it is impossible to confidently state their efficacy in TRD when used in combination with more modern antidepressants, such as SSRIs, due to a lack of data in this regard (Connolly and Thase, 2011). Given these issues, the troublesome side-effect profile of lithium treatment and its narrow therapeutic index, lithium is no longer widely used as an augmentation agent in TRD (Thase, 2004). The available evidence indicates that thyroid hormones, such as triiodothyronine (T_3) are essentially

equivalent to lithium in terms of their efficacy as augmenters of antidepressant activity, and therefore may be considered a preferable option given their generally better tolerability and greater ease of administration (Connolly and Thase, 2011). Additional randomised, placebo controlled trials are needed in order to evaluate the efficacy of lithium or thyroid hormone in patients who have not responded to first-line SSRI treatment (Connolly and Thase, 2011).

Atypical antipsychotic drugs, such as olanzapine, risperidone, quetiapine and aripiprazole, have also been reported to be effective augmentation agents in major depressive disorder (Nelson and Papakostas, 2009; Connolly and Thase, 2011). Atypical antipsychotics are pharmacologically diverse, with each drug having a distinct and complex set of receptor affinities involving dopaminergic and serotonergic receptors, in addition to varying effects on noradrenergic, histaminergic and cholinergic systems (Papakostas and Shelton, 2008).The proposed neuropharmacological rationale underlying the use of antipsychotic drugs as adjunctive agents in TRD involves their effects on various serotonin receptors, in particular antagonism of the 5-HT_2 receptor (Papakostas and Shelton, 2008). A meta-analysis revealed that these agents are equally efficacious as each other as adjunctive agents in TRD (Nelson and Papakostas, 2009). Moreover, the evidence supporting their efficacy is more robust than that of lithium or T_3 (Nelson and Papakostas, 2009). However, these atypical antipsychotic agents are associated with an increased risk of discontinuation due to adverse events, meaning that other strategies are often preferred (Nelson and Papakostas, 2009). The evidence supporting the efficacy other putative augmentation agents, such as buspirone, pindolol and psychostimulants is less than convincing (Connolly and Thase, 2011).

In addition to augmentation, other commonly used strategies in cases of failure to respond to initial antidepressant treatment involve switching to another antidepressant drug or concurrent treatment with a combination of different antidepressants. While the switching approach no doubt represents a good clinical option, there is insufficient data to suggest that any antidepressant has a unique therapeutic advantage compared to others in SSRI non-response (Connolly and

Thase, 2011). Additional studies are needed to address this question. Similarly, the use of antidepressant combination therapy in non-response is understudied, although available data suggest that addition of mirtazapine or mianserin to SSRI treatment may prove to be useful in this regard (Connolly and Thase, 2011).

As mentioned in Section 1.4.1, increasing recent evidence suggests that sub-anaesthetic doses of ketamine may represent a potentially rapid and effective option in TRD (Berman *et al.*, 2000; Zarate *et al.*, 2006). However, certain questions remain to be answered before this approach is adopted into widespread clinical practice (Rush, 2013). Given the restrictive inclusion criteria employed in studies to date, whether or not these promising findings will generalize to wider populations of depressed patients remains unclear. Moreover, the medium-to-long term management required to maintain remission in ketamine-treated patients is not yet known. The abuse potential and side effects associated with ketamine also represent substantial drawbacks. Identification of the molecular pathways responsible for the antidepressant action of ketamine and the potential development of more specific, non-psychomimetic, drugs targeting these pathways offers hope for the future (Cryan and O'Leary, 2010). Promisingly, a novel NMDAR glycine-site functional partial agonist, GLYX-13, has been reported to elicit antidepressant-like effects in rodent models of depression, without exhibiting substance abuse-related or sedative side effects associated with ketamine, and is now undergoing Phase II clinical development programme for TRD (Burgdorf *et al.*, 2013).

Other, non-pharmacological, strategies to combat TRD include vagus nerve stimulation, deep brain stimulation in the subgenual cingulate region of the brain, transcranial magnetic stimulation and electroconvulsive therapy(Holtzheimer and Mayberg, 2011). These approaches are reserved for severe cases of TRD, and vary in terms of their efficacy, with none representing a panacea in the treatment of depression (Holtzheimer and Mayberg, 2011).

In summary, TRD remains a considerable clinical problem. Despite the limitations of the monoamine hypothesis of depression, attempts to develop improved

pharmacological treatment strategies based on novel mechanisms have yet to bear fruit (Holtzheimer and Mayberg, 2011). Further research is required to attain a greater understanding of the reasons why some patients respond to antidepressant treatment while others do not, and to develop novel approaches to overcome TRD.

1.4.5. Animal models of depression and antidepressant-like activity

One of the major challenges in elucidating the neurobiological mechanisms underlying depression and in developing novel treatment strategies for this disorder is the generation of satisfactory *in vivo* model systems in which to explore these issues (Cryan and Holmes, 2005; O'Leary and Cryan, 2013). Due to practical and ethical limitations, conducting studies in humans to further our knowledge of the neurobiology of depression is difficult. Thus, translational animal models are vital to develop a greater understanding of the disorder (Berton *et al.*, 2012; O'Leary and Cryan, 2013). Furthermore, owing to the prohibitively large cost of clinical trials in pharmaceutical development, it is essential to have valid preclinical animal models to evaluate the potential efficacy of novel antidepressants prior to taking the decision to progress to clinical studies (Cryan and Holmes, 2005).

Despite difficulties in reproducing a complex human neuropsychiatric disorder like depression in lower species, it is possible to model certain symptoms associated with human depression in rodents (Table 1.6). For example, symptoms such as anhedonia, weight/appetite changes, sleep disturbances and disrupted cognitive performance can all be modelled in rodents. More complex psychological symptoms, such as suicidal thoughts or feelings of worthlessness/guilt, cannot be modelled, however (Cryan and Holmes, 2005). Thus, it is not possible to fully recapitulate human depression in rodents, and it is necessary to avoid the pitfall of anthropromorphization of emotion-related behaviours in such lower species, especially in light of the substantial differences in brain anatomy between humans and rodents (Cryan and Holmes, 2005; O'Leary and Cryan, 2013).

Moreover, the symptomatic heterogeneity of depression needs to be borne in mind when discussing animal models of depression (Berton *et al.*, 2012). Anhedonia, for

example, is only present in approximately 50% of depressed patients (Berton *et al.*, 2012). An animal model of depression involves an inducing manipulation, such as stress, resulting in a behavioural or neurobiological readout (O'Leary and Cryan, 2013). Thus, animal 'models' of depression which are based on particular individual behaviours reflecting depressive symptomatology, such as anhedonia, may be more accurately considered as 'tests' of depressive-like behaviours or of antidepressant-like activity rather than models *per se* (O'Leary and Cryan, 2013).

Despite their limitations, the use of rodent models of depression, in particular those involving transgenic mice, have helped to facilitate investigations of systems thought to be involved in depression (Cryan and Mombereau, 2004; Cryan and Holmes, 2005). The minimum requirements for a valid animal model of depression, as proposed by McKinney and Bunney (1969), are that: the symptomatology or manifestations are 'reasonably analogous' to human depression; behavioural changes are amenable to objective monitoring; behavioural changes are reversible by treatment with clinically effective antidepressant drugs; the model is reproducible between investigators. While these requirements act as a worthwhile guide in the development of animal models of depression, the need for behavioural changes to be reversible by clinically effective antidepressant treatments may have resulted in a circular situation whereby these models are primarily effective in the identification of novel treatments which act via the same mechanisms as already available antidepressants (Krishnan and Nestler, 2008). This may, in turn, have contributed to difficulties in successfully identifying novel treatment approaches. Furthermore, many compounds which have displayed antidepressant-like activity in rodent models of depression have not proven to be clinically useful (O'Leary and Cryan, 2013). Nonetheless, animal models of depression and antidepressant-like activity represent valuable research tools, and several such models are in use today (Table 1.9).

Two of the most commonly used and conceptually similar, but not synonymous, rodent models of depression and antidepressant-like activity are the forced swim test (FST, which can be performed in rats or mice) and the tail suspension test (TST,

which is generally only performed in mice) (Cryan and Mombereau, 2004; Cryan and Holmes, 2005; O'Leary and Cryan, 2013). These so-called 'behavioural despair' tests, in which rodents are placed in an inescapable cylinder of tepid water or suspended upside-down by the tail, respectively, are based on the principle that the rodents will initially engage in vigorous, but futile, escape-oriented movements, followed by increasing bouts of passive immobility (Cryan and Holmes, 2005). Administration of various clinically effective antidepressant treatments (both pharmacological and non-pharmacological) prior to either the FST or TST prolong the duration of escape-oriented activity, with a corresponding reduction in passive immobility (Cryan and Holmes, 2005). Furthermore, factors which are implicated in depression, including genetic predisposition and exposure to stress, alter immobility behaviour in these tests (Cryan and Mombereau, 2004; Cryan and Holmes, 2005).

Originally described as 'behavioural despair' (Porsolt *et al.*, 1977a; Porsolt *et al.*, 1977b), the precise meaning of immobility and its relevance to depression in these tests is controversial (Cryan and Mombereau, 2004). Given that immobility in the TST or FST appears to be due to an inability or reluctance to maintain effort, as opposed to a manifestation of generalised hypoactivity, it is tempting to speculate that it may be analogous to psychomotor impairments commonly seen in depressed patients (as listed in Table 1.6) (Cryan and Holmes, 2005). Another source of contention in relation to the FST and TST is the criticism that they are sensitive to acute antidepressant administration, rather than the chronic treatment required to elicit a clinical response (as discussed in Section 1.4.2), thereby suggesting that they may not act via the same long-term adaptive mechanisms that result in antidepressant effects in humans (Cryan and Holmes, 2005). Notwithstanding these criticisms, the FST and TST remain useful tools in depression-related research, in particular when it comes to assessing strategies to increase the pharmacodynamic activity of existing antidepressant treatments.

While the FST and TST are similar in their underlying principles and the main behavioural readout, there are some key differences between the two paradigms

which warrant discussion (Cryan *et al.*, 2005a). The TST avoids possible confounding contribution of hypothermic exposure and swimming capability (which can be an issue for some mouse strains) as encountered in the FST. Furthermore, the pattern of immobility differs between the two tests: in the FST animals engage in rapid bouts of active behaviour, with immobility only emerging after the second minute of the test. Contrastingly, in the TST animals can become immobile much earlier during testing, but do not remain immobile for periods as long as those encountered in the FST (Cryan *et al.*, 2005a). Moreover, the two tests offer differential sensitivity and divergent dose-response curves: whereas the FST may not be consistently sensitive in detecting SSRI activity, the TST generally is (Cryan *et al.*, 2005a). Furthermore, genetic knockout of the $GABA_B$ receptor induces an antidepressant effect in the FST, but not the TST (Mombereau *et al.*, 2004).

Other animal models of depression include the learned helplessness (LH) and chronic mild stress (CMS) models, both of which are based on the concept of maladaptive coping responses to uncontrollable stresses (Cryan and Holmes, 2005; O'Leary and Cryan, 2013), and the olfactory bulbectomy (OB) model (Song and Leonard, 2005). Chronic treatment with antidepressant drugs reverses some of the behavioural deficits in these models, thus providing pharmacological validity (Cryan and Holmes, 2005; Song and Leonard, 2005). In particular, the CMS and OB models result in behavioural, neurochemical, neuroimmune and neuroendocrine alterations resembling those seen in depressed patients, some of which are responsive only to chronic, but not acute, antidepressant treatment (Cryan and Holmes, 2005; Song and Leonard, 2005).

Additional paradigms, such as the sucrose preference test or intracranial self-stimulation can be employed to investigate depression-related behaviours (Cryan and Holmes, 2005). These tests are useful in assessing anhedonic aspects of animal models of depression, such as LH, CMS and OB listed above.

Table 1.9: Current rodent models used in depression research (adapted from O'Leary and Cryan, 2013)

Model	Description
Acute stress-based models	
Forced swim test (FST)	When placed in an inescapable container of water, rats and mice will adopt an immobile posture; the duration of immobility is reduced by antidepressant treatment
Modified FST	As per FST, but swimming and climbing behaviours are assessed, in addition to immobility. Serotonergic antidepressants increase swimming behaviour, whereas noradrenergic antidepressants increase climbing behaviour. Most commonly employed in rats.
Tail suspension test (TST)	When suspended from a bar by the tail, mice will adopt an immobile posture. Like the FST, the duration of immobility is reduced by antidepressant treatment.
Chronic stress-based models	
Maternal separation	When separated from their mother during early postnatal life, rodents (mainly rats) can develop depression-like phenotypes in adulthood.
Prenatal stress	Exposure to a stressor during pregnancy induces depression-like behaviour and persistent physiological changes in the offspring.
Unpredictable chronic mild stress	Rodents are exposed to a variety of unpredictable stressors, resulting in depressive-like behaviours, which are prevented by antidepressant treatment.
Social defeat stress	Exposure to a novel, aggressive counterpart on a daily basis, with repeated defeat, evokes depression-like behaviours in the test subject. In particular, the test subject displays social avoidance behaviours when put in contact with an unfamiliar counterpart, and this is reversed by chronic antidepressant treatment
Learned helplessness	Rodents subjected to repeated inescapable shocks subsequently do not escape when given the opportunity, an effect reduced by chronic antidepressant treatment.
Genetic predisposition studies	
Selective breeding	Independent lines of mice and rats that differ in depression-like phenotype have been generated by selective breeding based on depressive-like behaviours, for example Flinders sensitive line (FSL) rats and Rouen helpless mice
Strain differences	Various strains of mice and rats have been reported to exhibit differential responses in various models of depression and antidepressant-like activity
Directed gene targeting	Genetically modified mice have been generated with disrupted expression of proteins thought to play a role in depression

Model	Description
Dysfunction of limbic circuitry	
Olfactory bulbectomy (OB)	Removal of the olfactory bulbs in rodents results in various neurochemical and behavioural alterations, which are reversed by chronic antidepressant treatment.
Focal lesions	Transient inactivation of the rat infralimbic cortex has antidepressant-like effects
Optogenetics	Inhibition or activation of specific groups of neurons in limbic areas of the brain have differential effects on depression-like behaviours
Other	
Drug withdrawal	In rodents, amphetamine withdrawal is characterised by anhedonia, learned helplessness and increased immobility in the FST and TST.
Neonatal drug treatment	Neonatal administration of clomipramine induces various depression-like behaviours in adulthood

1.4.6. P-gp expression and activity in depression

A PET study using ^{11}C-verapamil has shown that P-gp function is increased in patients with major depression receiving antidepressant treatment (de Klerk *et al.*, 2009). Using this technique, cerebral volume of distribution of ^{11}C-verapamil, which is a transported P-gp substrate, was used as a surrogate measure of P-gp function. The reason for increased P-gp function in medicated depressed patients has not been fully elucidated, but there are several potential explanations that warrant further investigation.

Firstly, it may be due to P-gp induction by chronic antidepressant treatment itself, and therefore not be related to the disease *per se*, as it is known that treatment with xenobiotics can increase P-gp expression (Bauer *et al.*, 2004; Narang *et al.*, 2008). This theory is supported by both *in vitro* and *in vivo* studies demonstrating induction of P-gp by the antidepressant venlafaxine (Ehret *et al.*, 2007; de Klerk *et al.*, 2010).

Secondly, various cytokines increase the expression and activity of P-gp *in vitro* (Bauer *et al.*, 2007; Yu *et al.*, 2007; Liptrott *et al.*, 2009) and immune activation, with correspondingly increased circulating cytokine levels, has been widely reported in at least a subset of depressed patients (Schiepers *et al.*, 2005; Dinan, 2009). Therefore, immune activation may contribute to the increased P-gp function observed in medicated depressed patients. However, P-gp activity, as measured by the cerebral volume of distribution of ^{11}C-verapamil, was reduced in rats which were subjected to chronic foot-shock stress as a model of human depression (de Klerk *et al.*, 2010). This increase in the cerebral volume of distribution of ^{11}C-verapamil may be reflective of stress-mediated disruption of the BBB through non-P-gp-mediated mechanisms, however, as no other marker was used to verify the integrity of the BBB independent of P-gp. In addition, the translational value of this rat model to processes ongoing at the BBB in depressed patients is unclear.

Finally, functional SNPs in the *ABCB1* gene may explain the altered P-gp activity observed in depressed patients. Indeed, certain uncommon polymorphisms in *ABCB1* were linked to the severity of depressive symptoms in a cohort of Taiwanese

depressed patients (Lin *et al.*, 2011). The functional impact of these particular polymorphisms has not yet been characterised, however. It would be of interest to conduct a PET study in a similar group of patients to determine if BBB P-gp function is related to genotype.

1.5. Interactions between P-gp and antidepressant drugs at the BBB

Antidepressant drugs need to penetrate the BBB to reach their site of action within the brain. Therefore, it has been suggested that drug efflux by P-gp at the BBB may contribute to the high prevalence of TRD. Indeed, many antidepressant drugs interact with P-gp *in vitro* and *in vivo*, both as substrates and inhibitors. These interactions have been postulated to be of clinical importance, as P-gp efflux would limit the distribution of these drugs in to the brain, thereby potentially resulting in sub-therapeutic target-site concentrations (Loscher and Potschka, 2005b) (Figure 1.13). Interestingly, it has also been hypothesized that inhibition of P-gp may, in fact, be involved in the mechanism of action of antidepressant drugs (Pariante *et al.*, 2004), as discussed in detail in Section 1.5.7.

1.5.1. *In vitro* studies investigating the transport of antidepressants by P-gp

Assays to identify P-gp inhibitors are generally more cost-effective and less labour-intensive than studies aimed at identifying transported P-gp substrate. Therefore, the majority of *in vitro* studies investigating interactions between antidepressant drugs and-P-gp to date have focused on P-gp inhibition by antidepressants. However, a small number of studies using bidirectional transcellular transport assays (Rochat *et al.*, 1999; Mahar Doan *et al.*, 2002; Faassen *et al.*, 2003; El Ela *et al.*, 2004; Maines *et al.*, 2005), the ATPase assay (Ejsing *et al.*, 2006; Wang *et al.*, 2008) or both (Feng *et al.*, 2008) have been conducted.

Table 1.10 presents a summary of the available *in vitro* data on antidepressants as P-gp substrates. Column 2 indicates the conclusions drawn by the studies' authors, and it is important to be aware that the interpretation of results may differ between groups.

Table 1.10: *In vitro* studies investigating if various antidepressants are substrates of P-gp

Antidepressant	P-gp substrate / interaction?	Transport ratio	ATPase K_m (µM)	Model	Ref
Bidirectional Transport Studies					
Amitriptyline	N	1.34	n/a	MDCK-MDR1	Mahar Doan *et al.*, 2002
	Y	4.51	n/a	Caco-2	Faassen *et al.*, 2003
Citalopram	N [1]	1.6	n/a	MDCK-MDR1	Feng *et al.*, 2008
	N [2]	1.2	n/a	MDCK-mdr1a	Feng *et al.*, 2008
	N	1.1	n/a	BMEC monolayer	Rochat *et al.*, 1999
Clomipramine	N	1.42	n/a	MDCK-MDR1	Mahar Doan *et al.*, 2002
Desipramine	N	1.03	n/a	MDCK-MDR1	Mahar Doan *et al.*, 2002
	Y	3.98[3]	n/a	Caco-2	Faassen *et al.*, 2003
Doxepin	N	1.15	n/a	MDCK-MDR1	Mahar Doan *et al.*, 2002
Fluoxetine	N	1.18	n/a	MDCK-MDR1	Mahar Doan *et al.*, 2002
	N [1]	0.5	n/a	MDCK-MDR1	Feng *et al.*, 2008
	N [2]	0.6	n/a	MDCK-mdr1a	Feng *et al.*, 2008
Fluvoxamine	N	1.2	n/a	MDCK-MDR1	Mahar Doan *et al.*, 2002
	N [1]	0.9	n/a	MDCK-MDR1	Feng *et al.*, 2008
	N [2]	0.8	n/a	MDCK-mdr1a	Feng *et al.*, 2008
	Y	1.26	n/a	Caco-2	El Ela *et al.*, 2004
Imipramine	N	1.05	n/a	MDCK-MDR1	Mahar Doan *et al.*, 2002
	Y	6.04	n/a	Caco-2	Faassen et al., 2003
Nortriptyline	N	1.39	n/a	MDCK-MDR1	Mahar Doan *et al.*, 2002
	N [1]	1.1	n/a	MDCK-MDR1	Feng *et al.*, 2008
	N [2]	0.9	n/a	MDCK-mdr1a	Feng *et al.*, 2008
Paroxetine	N [1]	1	n/a	MDCK-MDR1	Feng *et al.*, 2008
	N [2]	1	n/a	MDCK-mdr1a	Feng *et al.*, 2008

Antidepressant	P-gp substrate / interaction?	Transport ratio	ATPase K_m (µM)	Model	Ref
Paroxetine (contd)	N	n/a	n/a	B(ovine)R(etinal)EC	Maines *et al.*, 2005
Protriptyline	Y	2	n/a	MDCK-MDR1	Mahar Doan *et al.*, 2002
Sertraline	N [1]	0.4	n/a	MDCK-MDR1	Feng *et al.*, 2008
	N [2]	0.5	n/a	MDCK-mdr1a	Feng *et al.*, 2008
Trazodone	N	0.94	n/a	MDCK-MDR1	Mahar Doan *et al.*, 2002
Trimipramine	N	0.92	n/a	MDCK-MDR1	Mahar Doan *et al.*, 2002
Venlafaxine	N [1]	0.9	n/a	MDCK-MDR1	Feng *et al.*, 2008
	N [2]	1	n/a	MDCK-mdr1a	Feng *et al.*, 2008
ATPase assay					
Bupropion	N	n/a	2676	ATPase assay	Wang *et al.*, 2008
→*EB*	N	n/a	109.3	ATPase assay	Wang *et al.*, 2008
→*HB*	N	n/a	318.2	ATPase assay	Wang *et al.*, 2008
→*TB*	N	n/a	2066	ATPase assay	Wang *et al.*, 2008
Citalopram	Low	n/a	441 ± 140.1	ATPase assay	Feng *et al.*, 2008
Fluoxetine	Low	n/a	51.6 ± 11.81	ATPase assay	Feng *et al.*, 2008
Fluvoxamine	Low	n/a	180 ± 35.7	ATPase assay	Feng *et al.*, 2008
Nortriptyline	Y	n/a	257.6	ATPase assay	Ejsing *et al.*, 2006
	Low	n/a	56.0 ± 15.73	ATPase assay	Feng *et al.*, 2008
Paroxetine	Moderate	n/a	26.2 ± 1.02	ATPase assay	Feng *et al.*, 2008
Sertraline	High	n/a	9.4 ± 1.45	ATPase assay	Feng *et al.*, 2008
	Y	n/a	4.7	ATPase assay	Wang *et al.*, 2008
→***Desmethylsert***	Y	n/a	6.5	ATPase assay	Wang *et al.*, 2008
Venlafaxine	N	n/a	Neg	ATPase assay	Feng *et al.*, 2008

K_m = Michaelis constant (substrate concentration at which the reaction rate is half of its maximum value)

[1, 2] Cut-off for significance: [1] 1.7 or [2] 1.5 (based on retrospective statistical analysis of inter-week system variability control data)

[3] Note very high donor concentration (1 mM); → *denotes a metabolite*

Of the antidepressants tested in transcellular transport assays to date, only protriptyline has been identified as a modest P-gp substrate, while conflicting findings have been reported in relation to amitriptyline, imipramine and fluvoxamine (Mahar Doan *et al.*, 2002; Faassen *et al.*, 2003; El Ela *et al.*, 2004; Feng *et al.*, 2008). However, the studies reporting positive findings in relation to amitriptyline, imipramine and fluvoxamine involved the use of the Caco-2 cell line, which expresses several other efflux and uptake transporters in addition to P-gp (Taipalensuu *et al.*, 2001). Further experiments, to confirm that P-gp was the transporter responsible for the elevated TR observed in Caco-2 cells, were not conducted (Faassen *et al.*, 2003; El Ela *et al.*, 2004), thereby rendering the findings in relation to P-gp inconclusive. Similarly, there were limitations associated with the studies reporting negative findings. For example, it has recently been shown that transfection with *ABCB1* in MDCK-MDR1 cells reduces the expression of endogenous canine P-gp relative to the wildtype MDCK cells (Kuteykin-Teplyakov *et al.*, 2010). This finding may have implications for the interpretation of results from many of the studies reporting negative findings, which were based on the assumption that endogenous drug transporter expression would be the same for wild-type and transfected cells.

In studies involving the ATPase assay, the SSRIs sertraline and paroxetine were identified as high- and moderate-affinity P-gp substrates, respectively, while the Michaelis constant (K_m) values for nortriptyline and fluoxetine narrowly exceeded the threshold set for moderate-affinity (Feng *et al.*, 2008). However, the ATPase assay does not determine if compounds are actually transported by P-gp. Thus, based on the available data from *in vitro* studies, it seems that some antidepressants interact with P-gp *in vitro*, but none have been robustly identified as high affinity P-gp substrates which are subjected to P-gp mediated transport in bidirectional transcellular transport assays.

1.5.2. *In vitro* studies investigating the inhibition of P-gp by antidepressants

Several antidepressants inhibit P-gp *in vitro* (Table 1.11). In Table 1.11, it is worth noting that the data in the second column represent the conclusions drawn by the specific authors of the individual studies. Again, it must be noted that the interpretation of results may differ from group to group.

Interestingly, the level of P-gp inhibition observed for several of these antidepressants was similar to that of established P-gp inhibitors, such as verapamil and quinidine (Szabo *et al.*, 1999; Weiss *et al.*, 2003; Feng *et al.*, 2008). For example, the concentration required to reduce P-gp activity by 50% (IC_{50}) for paroxetine (29.8µM) and sertraline (31.8 µM) is similar to that of quinidine (33.8 µM) (Weiss *et al.*, 2003). It should be noted, however, that these antidepressant concentrations are 250- and 500-fold higher than normal therapeutic plasma levels for paroxetine and sertraline respectively (Weiss *et al.*, 2003). However, post-mortem studies have demonstrated high brain-to-plasma concentration ratios for certain antidepressants (Pariante, 2008), and neuroimaging studies of fluorine-containing antidepressants have revealed steady-state brain concentrations in the micromolar range (Bolo *et al.*, 2000). Moreover, P-gp inhibition by sertraline has been demonstrated *in vivo* (Wang *et al.*, 2006a). Nonetheless, it remains uncertain if P-gp-inhibitory concentrations of paroxetine or sertraline are reached at the BBB in clinical use.

Table 1.11: *In vitro* studies investigating if various antidepressants are inhibitors of P-gp

Antidepressant	P-gp inhibitor?	IC_{50} (µM)	Model	Ref
Amitriptyline	Y	n/a	R123 uptake in Caco-2 cells	Ibrahim *et al*., 2000
	Y	n/a	R123/daunorubicin cellular uptake	Szabo *et al*., 1999
	Y	n/a	R123 efflux	Varga *et al*., 1996
Citalopram	Y	52.5	Calcein-AM transport in MDCK-MDR1 cells	Feng *et al*., 2008
Clomipramine	Y	n/a	R123 uptake in Caco-2 cells	Ibrahim *et al*., 2000
	Y	n/a	Cell-line susceptibility to P-gp-substrate cytotoxics	Jaffrezou *et al*., 1995
	Y	n/a	Cell-line susceptibility to P-gp-substrate cytotoxics	Merry *et al*., 1991
Desipramine	Y	n/a	R123 uptake in Caco-2 cells	Ibrahim *et al*., 2000
	Y	n/a	Cell-line susceptibility to P-gp-substrate cytotoxics	Jaffrezou *et al*., 1995
	Y	n/a	R123/daunorubicin cellular uptake	Szabo *et al*., 1999
Doxepin	Y	n/a	R123 uptake in Caco-2 cells	Ibrahim *et al*., 2000
	Y	n/a	R123/daunorubicin cellular uptake	Szabo *et al*., 1999
Fluoxetine	Y	31	Calcein-AM transport in MDCK-MDR1 cells	Feng *et al*., 2008
	Y	n/a	Digoxin uptake in Caco-2 cells	Pariante *et al*., 2003b
	Y	115.5 ± 11.7	Calcein-AM transport in L-MDR1 and pBCEC cells	Weiss *et al*., 2003
Fluvoxamine	N	> 100	Calcein-AM transport in MDCK-MDR1 cells	Feng *et al*., 2008
	?	146.7 ± 1.95	Talinolol transport in Caco-2 cells	El Ela *et al.,* 2004
Imipramine	Y	n/a	R123 uptake in Caco-2 cells	Ibrahim *et al*. 2000
	Y	n/a	Cell-line susceptibility to P-gp-substrate cytotoxics	Jaffrezou *et al*. 1995
	Y	n/a	R123/daunorubicin cellular uptake	Szabo *et al*. 1999

Antidepressant	P-gp inhibitor?	IC_{50} (μM)	Model	Ref
Maprotiline	Y	n/a	R123/daunorubicin cellular uptake	Szabo *et al*. 1999
Mianserin	Y	n/a	Cell-line susceptibility to P-gp-substrate cytotoxics	Jaffrezou *et al*. 1995
Nefazodone	Y	4.7	R123 uptake in Caco-2 cells	Stormer *et al*. 2001
Nortriptyline	N	> 100	Calcein-AM transport in MDCK-MDR1 cells	Feng *et al*. 2008
	N	n/a	R123 uptake in Caco-2 cells	Ibrahim *et al*. 2000
Paroxetine	Y	27.5	Calcein-AM transport in MDCK-MDR1 cells	Feng *et al*. 2008
	Y	n/a	[3H]Taxol uptake in bovine retinal endothelial cells	Maines *et al*. 2005
	Y	29.8 ± 11.1	Calcein-AM transport in L-MDR1 and pBCEC cells	Weiss *et al*. 2003
Protriptyline	Y	n/a	R123 uptake in Caco-2 cells	Ibrahim *et al*. 2000
Sertraline	Y	30	Calcein-AM transport in MDCK-MDR1 cells	Feng *et al*. 2008
	Y	31.8 ± 2.8	Calcein-AM transport in L-MDR1 and pBCEC cells	Weiss *et al*. 2003
Trazodone	N	n/a	R123 uptake in Caco-2 cells	Stormer *et al*. 2001
Trimipramine	Y	n/a	R123 uptake in Caco-2 cells	Ibrahim *et al*. 2000
	Y	n/a	Cell-line susceptibility to P-gp-substrate cytotoxics	Jaffrezou *et al*. 1995
	Y	n/a	R123/daunorubicin cellular uptake	Szabo *et al*. 1999
Venlafaxine	N	> 100	Calcein-AM transport in MDCK-MDR1 cells	Feng *et al*. 2008
Selected prototypical P-gp inhibitors				
Quinidine	Y	30.2	Calcein-AM transport in MDCK-MDR1 cells	Feng *et al*. 2008
	Y	33.8 ± 11.9	Calcein-AM transport in L-MDR1 and pBCEC cells	Weiss *et al*. 2003
Verapamil	Y	18.1	Calcein-AM transport in MDCK-MDR1 cells	Feng *et al*. 2008
	Y	18.9 ± 4.2	Calcein-AM transport in L-MDR1 and pBCEC cells	Weiss *et al*. 2003

1.5.3. Limitations of *in vitro* studies investigating interactions between P-gp and antidepressant drugs

It is important to consider the limitations of these *in vitro* studies investigating interactions between P-gp and antidepressant drugs; different studies have used the same assay for the same compound and reported contrasting results. Furthermore, different groups offer different interpretations of results, with contrasting cut-off values for significance.

For example, the determined K_m value for nortriptyline differed greatly between two studies using the same assay (ATPase), 257.6 µM (Ejsing *et al.*, 2006) versus 56 ± 15.73 µM (Feng *et al.*, 2008). Furthermore, the interpretation of these results conflicted between the two groups. Despite the fact that a lower K_m value correlates with a stronger interaction, Ejsing and co-workers concluded that their findings confirmed an interaction which may be of clinical significance between nortriptyline and P-gp, while Feng and colleagues suggest that any such interaction is of minimal significance as they classified nortriptyline as a low-affinity P-gp-substrate. Similarly discrepant findings were obtained in relation to the P-gp-substrate status of amitriptyline, imipramine and fluvoxamine by different groups using transwell assay systems (Mahar Doan *et al.*, 2002; Faassen *et al.*, 2003; El Ela *et al.*, 2004; Feng *et al.*, 2008).

Additionally, in P-gp-inhibition studies there was a marked contrast in the reported IC_{50} values for fluoxetine, 31 µM (Feng *et al.*, 2008) *versus* 115.5 ± 11.7 µM (Weiss *et al.*, 2003), while reported values for paroxetine and sertraline were in quite close agreement between the same two studies. Thus, it can be difficult to draw definitive conclusions from *in vitro* P-gp studies due to the discrepancies in results obtained depending on assay used and numerous other potential confounding factors. As a result, there remains a demand for the development of a validated and highly reliable predictive screening model for the accurate identification of P-gp substrates and inhibitors *in vitro* (von Richter *et al.*, 2009).

In addition to the discordance in results obtained from different *in vitro* studies, there has been disagreement between *in vitro* and *in vivo* findings regarding the P-

gp-substrate status of certain antidepressant drugs. For example, citalopram (Rochat *et al.*, 1999; Feng *et al.*, 2008) and paroxetine (Maines *et al.*, 2005; Feng *et al.*, 2008) were found not to be P-gp-substrates in various cell culture models. However, *in vivo* studies using P-gp knockout mice have demonstrated increased brain/plasma concentration ratios in knockout mice relative to wild-type controls for both drugs (see Section 1.5.4), thus suggesting that they are P-gp substrates at the BBB *in vivo* (Uhr and Grauer, 2003; Uhr *et al.*, 2003; Doran *et al.*, 2005; Uhr *et al.*, 2008). These differences may be as a result of a failure of *in vitro* systems to adequately reflect the complexity of the BBB *in vivo,* and therefore highlight the difficulty in achieving acceptable correlations between *in vitro* and *in vivo* data in relation to drug transport across the BBB (Avdeef, 2011), thus calling the relevance of any of these *in vitro* assays into question. As P-gp extrudes its substrates directly from the inner leaflet of the cellular membrane bilayer, the properties of the membrane used in *in vitro* studies impact considerably on interactions between P-gp and the compound being tested (Romsicki and Sharom, 1999). Therefore, the use of epithelial cell lines such as Caco-2 and MDCK-MDR1 to predict interactions between drugs and P-gp expressed at the endothelial BBB *in vivo* may not be appropriate. To date, the use of BBB-derived endothelial cell lines in this regard has been limited, primarily due to the leakiness of such cell lines when cultured as monolayers *in vitro* (Avdeef, 2011). Alternatively, the discrepancies observed between *in vitro* and *in vivo* studies may highlight important species differences between substrate specificity of human (as measure in *in vitro* studies) and rodent P-gp, or false assumptions relating to results derived from knockout mice. Indeed, the relevance of marginally increased brain/plasma ratios in knockout mice, relative to controls, has been questioned (Feng *et al.*, 2008).

1.5.4. *In vivo* studies involving P-gp knockout rodents

Several studies using P-gp knockout mouse models, and a recent study using P-gp knockout rats (Bundgaard *et al.*, 2012), have determined that P-gp plays a major role in the brain penetration of several antidepressants (Table 1.12) (Uhr *et al.*, 2000; Uhr and Grauer, 2003; Uhr *et al.*, 2003; Grauer and Uhr, 2004; Doran *et al.*,

2005; Uhr *et al.*, 2007; Uhr *et al.*, 2008; Karlsson *et al.*, 2010; Karlsson *et al.*, 2011).It is important to note that the majority of studies using the P-gp knockout mouse models in this regard have involved acute antidepressant administration. As antidepressant drugs are administered chronically in clinical practice, studies investigating the impact of P-gp ablation on the brain distribution of antidepressant drugs and their metabolites following chronic administration would be of greater value.

Interestingly, many of the identified P-gp-substrate-antidepressants are structurally unrelated and come from a variety of drug classes: including SSRIs, TCAs and SNRIs. Not all drugs from the same class behave similarly, however: sertraline, another SSRI for example, is not a P-gp substrate (Doran *et al.*, 2005).

Amitriptyline was found to have significantly higher brain concentrations in mice deficient in P-gp than in wild-type controls one hour after acute administration (Uhr *et al.*, 2000). However, later studies determined that this effect was transient and dependent on the dosing regimen and/or the route of administration (Grauer and Uhr, 2004; Uhr *et al.*, 2007). Thus, the relevance of P-gp to the CNS pharmacokinetics of amitriptyline following chronic administration and at steady state conditions is uncertain. While the exact reason for this phenomenon is unclear, a number of theories have been proposed (Uhr *et al.*, 2007). For example, it is postulated that amitriptyline may induce changes at the BBB itself or that P-gp may become oversaturated with time, as certain amitriptyline metabolites are also P-gp substrates. Furthermore the original study used single knockout *abcb1a*$^{(-/-)}$ mice (Uhr *et al.*, 2000), while subsequent studies used double knockout *abcb1ab*$^{(-/-)}$ mice (Grauer and Uhr, 2004; Uhr *et al.*, 2007). Thus, the contrasting findings of the two studies may be explained by differences between single knockout *abcb1a*$^{(-/-)}$ mice and double knockout *abcb1ab*$^{(-/-)}$ mice.

Similarly, there is also a lack of clarity in the literature regarding the status of fluoxetine as a P-gp substrate *in vivo* (Uhr *et al.*, 2000; Doran *et al.*, 2005). The original study, using single knockout *abcb1a*$^{(-/-)}$ mice, found no difference between the accumulation of fluoxetine in the brains of *abcb1a*$^{(-/-)}$ mice and wild type

controls (Uhr *et al.*, 2000). A subsequent study, however, found increased levels of fluoxetine in double knockout *abcb1ab*$^{(-/-)}$ mice relative to wild type controls (Doran *et al.*, 2005). Taken together, these data suggest that the brain pharmacokinetics of certain compounds may differ between single knockout *abcb1a*$^{(-/-)}$ mice and double knockout *abcb1ab*$^{(-/-)}$ mice. Indeed, a recent study has also reported that the BBB transport of glucocorticoids differs between single and double P-gp knockout mice (Mason *et al.*, 2012). Furthermore, the data from Uhr and co-workers is from a single time point (one hour) following fluoxetine administration (Uhr *et al.*, 2000); Doran and colleagues, on the other hand, took samples at various time points (Doran *et al.*, 2005). Therefore, the impact of P-gp on fluoxetine distribution may be time-dependent.

In addition to investigating the impact of P-gp ablation on venlafaxine pharmacokinetics, Karlsson and co-workers looked at differences in behavioural responses to subchronic venlafaxine treatment between wild-type and P-gp knockout mice (Karlsson *et al.*, 2011). Chronic treatment with venlafaxine affected behaviour in the open field, with increased time spent in the centre of the arena and a reduced number of rears associated with venlafaxine treatment. Interestingly, this pharmacodynamic effect was more pronounced in P-gp knockout mice than wild-type controls after 7 days of venlafaxine treatment. The difference between the two strains was attenuated after 9 days of treatment, which may have been due to habituation associated with repeated testing in the open field (Thiel *et al.*, 1999). While pharmacodynamic differences between P-gp knockout and wild-type mice have previously been demonstrated following treatment with the antipsychotic drug, risperidone (Kirschbaum *et al.*, 2008), this remains the only study demonstrating pharmacodynamic differences between the strains in response to treatment with an antidepressant drug, and for this reason it is important. However, the relevance of behaviour in the open field to antidepressant treatment is unclear, and future studies in this area should focus on behavioural pharmacodynamic readouts in established animal models of depression, such as the forced swim test and tail suspension test (Cryan and Holmes, 2005).

Table 1.12: *In vivo* studies in P-gp knock-out rodents investigating if various antidepressants are P-gp substrates at the BBB

Antidepressant	Dose	Class	Samples taken	Brain/Plasma Conc.			Sig	Model	Ref
				WT	KO	KO/WT			
Amitriptyline	10 mg/kg s.c.	TCA	30 min post-injection	8.1	8.0	1.0 [3]	ns	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	10 mg/kg s.c.	TCA	60 min post-injection	11.5	8.9	0.8 [3]	ns	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	10 mg/kg s.c.	TCA	120 min post-injection	11.7	16.0	1.4 [3,4]	*	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	10 mg/kg s.c.	TCA	240 min post-injection	12.8	12.0	0.9 [3]	ns	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	5 mg/kg i.p.	TCA	1 h post-injection	n/a	n/a	~ 1.9 [1,2]	*	female mdr1a$^{(-/-)}$ mice	Uhr *et al*., 2000
	10 mg/kg s.c. bd for 10 days	TCA	4 h after final dose	10.3	13.3	1.3 [3]	ns	male mdr1ab$^{(-/-)}$ mice	Grauer and Uhr, 2004
→ *E-OH- AMI*	n/a	metab	30 min post-AMI injection	1.4	3.8	2.7 [3]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	60 min post-AMI injection	1.8	4.2	2.4 [3]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	120 min post-AMI injection	2.1	5.1	2.4 [3]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	240 min post-AMI injection	1.9	5.0	2.7 [3,4]	*	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	1 h post-AMI injection	n/a	n/a	~ 3.1 [1,2]	*	male mdr1a$^{(-/-)}$ mice	Uhr *et al*., 2000

Antidepressant	Dose	Class	Samples taken	Brain/Plasma Conc.			Sig	Model	Ref
				WT	KO	KO/WT			
→ *E-OH- AMI (contd)*	n/a	metab	4 h after final AMI dose	1.6	2.2	1.3 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Grauer and Uhr, 2004
→ *Z-OH-AMI*	n/a	metab	30 min post-AMI injection	1.3	3.6	2.8 [3]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	60 min post-AMI injection	1.6	3.4	2.1 [3]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	120 min post-AMI injection	1.5	4.9	3.3 [3]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	240 min post-AMI injection	1.4	6.8	4.7 [3,4]	*	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	1 h post-AMI injection	n/a	n/a	~ 4.5 [1,2]	*	male mdr1a$^{(-/-)}$ mice	Uhr *et al*., 2000
	n/a	metab	4 h after final AMI dose	1.5	7.4	4.9 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Grauer and Uhr, 2004
→*E-OH-NOR*	n/a	metab	30 min post-AMI injection	n/a [5]	0.5	n/a [3,5]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	60 min post-AMI injection	n/a [5]	0.6	n/a [3,5]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	120 min post-AMI injection	0.5	1.0	2.2 [3]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	240 min post-AMI injection	0.6	1.9	3.2 [3,4]	*	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	1 h post-AMI injection	n/a	n/a	~ 2.8 [1,2]	*	male mdr1a$^{(-/-)}$ mice	Uhr *et al*., 2000

Antidepressant	Dose	Class	Samples taken	Brain/Plasma Conc.			Sig	Model	Ref
				WT	KO	KO/WT			
	n/a	metab	4 h after final AMI dose	0.5	3.7	8.0 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Grauer and Uhr, 2004
→Z-OH-NOR	n/a	metab	30 min post-AMI injection	n/a [5]	0.2	n/a [3,5]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al.*, 2007
	n/a	metab	60 min post-AMI injection	n/a [5]	0.2	n/a [3,5]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al.*, 2007
	n/a	metab	120 min post-AMI injection	0.2	0.5	2.5 [3]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al.*, 2007
	n/a	metab	240 min post-AMI injection	0.3	0.9	2.8 [3,4]	*	female mdr1ab$^{(-/-)}$ mice	Uhr *et al.*, 2007
	n/a	metab	1 h post-AMI injection	n/a	n/a	~ 2.3 [1,2]	*	male mdr1a$^{(-/-)}$ mice	Uhr *et al.*, 2000
	n/a	metab	4 h after final AMI dose	0.4	2.5	5.7 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Grauer and Uhr, 2004
Citalopram	1 mg/kg s.c.	SSRI	1 h post-injection	4.6	15.7	3.4 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Uhr and Grauer, 2003
	60 µg/day s.c	SSRI	After 11 days treatment	1.5	5.4	3.7	*	male mdr1ab$^{(-/-)}$ mice	Uhr *et al.*, 2008
	3 mg/kg s.c.	SSRI	Multiple time points	5.1	9.7	1.9	*	female mdr1ab$^{(-/-)}$ mice	Doran *et al.*, 2005
	5 mg/kg/day s.c. infusion	SSRI	After 3 days treatment	22	44	2.0	***	mdr1a$^{(-/-)}$ mice	Bundgaard *et al.*, 2012
	9 mg/kg/day s.c. infusion	SSRI	After 3 days treatment	10	33	3.3	***	mdr1a$^{(-/-)}$ rat	Bundgaard *et al.*, 2012

Antidepressant	Dose	Class	Samples taken	Brain/Plasma Conc.			Sig	Model	Ref
				WT	KO	KO/WT			
Doxepin	10 mg/kg s.c.	TCA	1 h post-injection	7.1	7.6	1.1 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2003
→*D-Doxepin*	n/a	metab	1 h post-DOX injection	1.9	2.4	1.3 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2003
Fluoxetine	1.5 mg/kg i.p	SSRI	1 h post-injection	n/a	n/a	~ 1.1 [1,2]	ns	male mdr1a$^{(-/-)}$ mice	Uhr *et al*., 2000
	3 mg/kg s.c.	SSRI	Multiple time points	12.0	18.0	1.5	*	female mdr1ab$^{(-/-)}$ mice	Doran *et al*., 2005
→*Norfluoxetine*	n/a	metab	1 h post-FLX injection	n/a	n/a	~ 1.1 [1,2]	ns	male mdr1a$^{(-/-)}$ mice	Uhr *et al*., 2000
Fluvoxamine	3 mg/kg s.c.	SSRI	Multiple time points	6.1	14.0	2.3	*	female mdr1ab$^{(-/-)}$ mice	Doran *et al*., 2005
Mirtazapine	1 mg/kg s.c.	NaSSA	1 h post-injection	3.1	3.0	1.0	ns	male mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2003
	60 µg/day s.c	NaSSA	After 11 days treatment	4.6	4.6	1.0	ns	male mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2008
Nortriptyline	3 mg/kg s.c.	TCA	Multiple time points	11.0	20.0	1.8	*	female mdr1ab$^{(-/-)}$ mice	Doran *et al*., 2005
	5 mg/kg s.c.	TCA	n/a	n/a	n/a	1.6	*	mdr1a$^{(-/-)}$ mice	Ejsing *et al*., 2006
→ *Nortriptyline*	n/a	metab	30 min post-AMI injection	2.8	2.6	0.9 [3]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	60 min post-AMI injection	3.1	4.9	1.6 [3]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007

Antidepressant	Dose	Class	Samples taken	Brain/Plasma Conc.			Sig	Model	Ref
				WT	KO	KO/WT			
→ *Nortriptyline (contd)*	n/a	metab	120 min post-AMI injection	4.8	7.3	1.5 [3]	n/a	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	240 min post-AMI injection	8.1	11.4	1.4 [3,4]	*	female mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2007
	n/a	metab	1 h post-AMI injection	n/a	n/a	~ 2.4 [1,2]	*	male mdr1a$^{(-/-)}$ mice	Uhr *et al*., 2000
	n/a	metab	4 h after final AMI dose	6.8	14.6	2.1 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Grauer and Uhr, 2004
Paroxetine	3 mg/kg s.c.	SSRI	Multiple time points	3.3	7.1	2.2	*	female mdr1ab$^{(-/-)}$ mice	Doran *et al*., 2005
	1 mg/kg s.c.	SSRI	1 h post-injection	1.9	3.0	1.6 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2003
Sertraline	3 mg/kg s.c.	SSRI	Multiple time points	24.0	27.0	1.1	ns	female mdr1ab$^{(-/-)}$ mice	Doran *et al*., 2005
Trimipramine	10 mg/kg s.c.	TCA	1 h post-injection	7.0	9.7	1.4 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Uhr and Grauer, 2003
→D-Trimipramine	n/a	metab	1 h post-TRIM injection	2.8	3.3	1.2 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Uhr and Grauer, 2003
Venlafaxine	3 mg/kg s.c.	SNRI	Multiple time points	4.2	7.7	1.8	*	female mdr1ab$^{(-/-)}$ mice	Doran *et al*., 2005
	5 mg/kg s.c.	SNRI	1 h post-injection	4.9	8.8	1.8 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2003
	300 µg /day s.c	SNRI	After 11 days treatment	3.7	6.5	1.8	*	male mdr1ab$^{(-/-)}$ mice	Uhr *et al*., 2008

Antidepressant	Dose	Class	Samples taken	Brain/Plasma Conc.			Sig	Model	Ref
				WT	KO	KO/WT			
Venlafaxine (contd)	10 mg/kg i.p	SNRI	1 h post-injection	4.1	8.9	2.2 [3,4]	**	male mdr1ab$^{(-/-)}$ mice	Karlsson *et al.*, 2010
	10 mg/kg i.p	SNRI	3 h post-injection	4.3	7.4	1.7 [3,4]	***	male mdr1ab$^{(-/-)}$ mice	Karlsson *et al.*, 2010
	10 mg/kg i.p	SNRI	6 h post-injection	3.7	5.5	1.5 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Karlsson *et al.*, 2010
	10 mg/kg i.p	SNRI	9 h post-injection	4.0	4.8	1.2 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Karlsson *et al.*, 2010
	10 mg/kg i.p bd for 10 days	SNRI	1 h after last injection	4.8	8.2	1.7 [3,4]	***	male mdr1ab$^{(-/-)}$ mice	Karlsson *et al.*, 2011
	15 mg/kg/day s.c. infusion	SSRI	After 3 days treatment	4.7	8.3	1.8	*	mdr1a$^{(-/-)}$ mice	Bundgaard *et al.*, 2012
	9 mg/kg/day s.c. infusion	SSRI	After 3 days treatment	3.3	6.3	1.9	**	mdr1a$^{(-/-)}$ rat	Bundgaard *et al.*, 2012
→D-Venlafaxine	n/a	metab	1 h post-VEN injection	1.4	2.3	1.7 [3,4]	*	male mdr1ab$^{(-/-)}$ mice	Uhr *et al.*, 2003
	n/a	metab	After 11 days VEN treatment	1.6	6.2	3.6	*	male mdr1ab$^{(-/-)}$ mice	Uhr *et al.*, 2008
	n/a	metab	1 h post-VEN injection	1.7	3.9	2.2 [3,4]	**	male mdr1ab$^{(-/-)}$ mice	Karlsson *et al.*, 2010
	n/a	metab	3 h post-VEN injection	5.0	12.0	2.4 [3,4]	**	male mdr1ab$^{(-/-)}$ mice	Karlsson *et al.*, 2010
	n/a	metab	6 h post-VEN injection	6.2	23.8	3.9 [3,4]	***	male mdr1ab$^{(-/-)}$ mice	Karlsson *et al.*, 2010

Antidepressant	Dose	Class	Samples taken	Brain/Plasma Conc.			Sig	Model	Ref
				WT	KO	KO/WT			
→*D-Venlafaxine (contd)*	n/a	metab	9 h post-VEN injection	5.3	15.9	3.0 [3,4]	**	male mdr1ab$^{(-/-)}$ mice	Karlsson *et al*., 2010
	n/a	metab	1 h after last VEN injection	1.8	3.5	1.9 [3,4]	***	male mdr1ab$^{(-/-)}$ mice	Karlsson *et al*., 2011

KO: P-gp knockout mice; KO/WT: ratio of brain/plasma ratios between KO and WT mice; metab: metabolite; NaSSA: noradrenergic and selective serotonergic antidepressant; SNRI: serotonin-noradrenaline reuptake inhibitor; SSRI: selective serotonin reuptake inhibitor; TCA: tricyclic antidepressant; WT: wild-type mice

→ denotes a metabolite

It is important to consider the inherent limitations of the P-gp knockout mouse model's utility in the assessment of the impact of P-gp mediated efflux on drug distribution into the brain. For example, the expression of BCRP mRNA in the cerebral microvessels of *abcb1a*$^{(-/-)}$ mice is increased threefold relative to wild-type animals (Cisternino *et al.*, 2004). Furthermore, this increase in BCRP mRNA expression is associated with a greater export of two of its substrates, prazosin and mitoxantrone, from the brain of *abcb1a*$^{(-/-)}$ mice versus wild-type controls (Cisternino *et al.*, 2004). Importantly, other ABC transporters, including BCRP, demonstrate overlapping drug specificities with P-gp (Litman *et al.*, 2001; Sharom, 2008), and the expression of ABC drug transporters at the BBB may become upregulated following exposure to xenobiotics (Miller, 2010). Therefore, potential upregulation of alternative drug efflux transporters, such as BCRP, in P-gp knockout mice must be considered before valid conclusions can be drawn regarding *in vivo* P-gp-drug interactions using this model.

In addition, following a comparison of *in vitro* and *in vivo* data for 23 compounds, it has been recommended that a threshold ratio of four-fold increase in brain/plasma drug concentration ratios between P-gp knockout and wild-type mice (KO:WT B/P ratio) should be set to assess P-gp-drug interactions *in vivo* (Feng *et al.*, 2008). For example, risperidone, despite having a KO:WT B/P ratio of 10, indicating that it is heavily subjected to P-gp efflux, is still a reasonably efficacious antipsychotic agent (Doran *et al.*, 2005). This might reflect the potency of risperidone, meaning that high brain concentrations are not required for it to exert its clinical effect. To date, no antidepressant drug has been shown to have a KO:WT B/P ratio greater than 3.6 (Table 1.12). Thus, it seems that no antidepressant has yet been identified as a high affinity transported P-gp substrate *in vivo*, and therefore the overall influence of P-gp efflux on the effectiveness of antidepressants has been questioned (Doran *et al.*, 2005). Furthermore, in many studies using knockout mice, brain antidepressant concentrations were not normalized against plasma levels (i.e. a brain/plasma concentration ratio was not used). Despite plasma drug concentrations being considerably higher in P-gp knockout than wild-type mice in some these studies, in certain cases with statistically significant differences in plasma drug levels between

the strains (Uhr *et al.*, 2003), brain drug concentrations were compared directly between strains (Uhr *et al.*, 2003; Uhr *et al.*, 2007). Thus statistically significant differences in brain drug concentrations identified between these two strains in such studies may have been partially due to variations in plasma pharmacokinetics, rather than solely as a result of altered drug transport across the BBB due to a lack of P-gp.

1.5.5. *In vivo* studies involving P-gp inhibition in wild-type rodents

Other *in vivo* studies have made use of established P-gp inhibitors, such as verapamil and cyclosporin A (CsA), to investigate if inhibition of P-gp results in altered antidepressant drug penetration in to the brain (Table 1.13).

Using this method, nortriptyline was shown to have significantly higher brain/serum ratios in rats treated with a P-gp-inhibitor than in untreated controls (Ejsing and Linnet, 2005). However, this increase was relatively small, equating to a c. 25% increase in the brain-serum ratio, compared to an 80% increase in the brains of P-gp knockout compared to wild-type mice (Doran *et al.*, 2005).

The use of whole brain in the analysis above may have obscured potentially greater region-specific central increases in drug concentration. In order to address this issue, the effect of P-gp inhibition on imipramine and desipramine concentrations in various brain regions was determined, and it was found that there was a region-specific effect (Clarke *et al.*, 2009). For example, the frontal cortex/serum imipramine concentration ratio was significantly increased to 2.4, compared to a non-significant ratio of 1.1 in the hypothalamus (Clarke *et al.*, 2009). Similarly for desipramine, a significant effect was observed in the frontal cortex but not for other brain areas (Clarke *et al.*, 2009). Thus, it seems that the effect of P-gp on drug penetration into the CNS is not uniform, and region-specific effects should be taken in to account when interpreting data in P-gp-interaction studies, as potentially significant results may be obscured or missed entirely.

Interestingly, it has been demonstrated that pharmacological induction of P-gp using any one of three different inducing agents (rifampicin, dexamethasone or 5-pregnene-3beta-ol-20-on-16alpha-carbonitrile) results in reduced brain distribution of risperidone and its active metabolite, 9-hydroxyrisperidone (Holthoewer *et al.*, 2010). Similar investigations in relation to antidepressant drugs would help to further clarify the role played by P-gp in their BBB transport.

A study evaluating pharmacokinetic interactions between the antipsychotic drug risperidone and the antidepressant sertraline in CF1 mice demonstrated P-gp inhibition by sertraline, resulting in significantly increased brain risperidone levels (Wang *et al.*, 2006a). This *in vivo* finding supports *in vitro* studies reporting P-gp inhibition by sertraline (Weiss *et al.*, 2003; Feng *et al.*, 2008). As the plasma sertraline levels achieved in this study approximate those observed in patients treated with the drug, this finding suggests that sertraline may induce clinically significant P-gp inhibitory effects in therapeutic use. Whether these preclinical findings can translate into therapeutic potential of P-gp inhibitors as adjunctive therapies in the treatment of schizophrenia remains to be determined.

Table 1.13: *In vivo* studies using P-gp inhibition to investigate if various antidepressants are P-gp substrates at the BBB

Antidepressant	Dose	Class	Species (strain)	P-gp inhibitor	Brain Region	Brain/Serum Conc.			Sig	Ref
						Control	P-gp I	P-gp I / Control		
Imipramine	15 mg/kg i.p.	TCA	Rat (Sprague Dawley)	Verapamil (20 mg/kg i.p.)	HYPO	21.5	23.6	1.1	ns	Clarke *et al.*, 2009
	15 mg/kg i.p.	TCA	Rat (Sprague Dawley)	Verapamil (20 mg/kg i.p.)	FC	11.9	28.4	2.4	*	Clarke *et al.*, 2009
	15 mg/kg i.p.	TCA	Rat (Sprague Dawley)	Verapamil (20 mg/kg i.p.)	HIPPO	17.4	26.0	1.5	*	Clarke *et al.*, 2009
	15 mg/kg i.p.	TCA	Rat (Sprague Dawley)	Verapamil (20 mg/kg i.p.)	BS	12.2	25.2	2.1	***	Clarke *et al.*, 2009
→ *Desipramine*	IMI 15 mg/kg i.p.	meta bolite	Rat (Sprague Dawley)	Verapamil (20 mg/kg i.p.)	HYPO	31.5	32.3	1.0	ns	Clarke *et al.*, 2009
	IMI 15 mg/kg i.p.	meta bolite	Rat (Sprague Dawley)	Verapamil (20 mg/kg i.p.)	HIPPO	13.8	15.1	1.1	ns	Clarke *et al.*, 2009
	IMI 15 mg/kg i.p.	meta bolite	Rat (Sprague Dawley)	Verapamil (20 mg/kg i.p.)	FC	13.2	19.0	1.4	**	Clarke *et al.*, 2009
	IMI 15 mg/kg i.p.	meta bolite	Rat (Sprague Dawley)	Verapamil (20 mg/kg i.p.)	BS	10.0	13.4	1.3	ns	Clarke *et al.*, 2009
Nortriptyline	5 mg/kg i.p.	TCA	Rat	Verapamil (50 mg/kg i.p.)	Whole brain	n/a	n/a	1.6	**	Ejsing *et al.*, 2006
	various i.p. (pooled)	TCA	Rat	Cyclosporin (200 mg/kg i.p.)	Whole brain	n/a	n/a	1.3	*	Ejsing *et al.*, 2006

Antidepressant	Dose	Class	Species (strain)	P-gp inhibitor	Brain Region	Brain/Serum Conc.			Sig	Ref
						Control	P-gp I	P-gp I / Control		
Nortriptyline (contd)	25 mg/kg p.o.	TCA	Rat (Wistar Hanover GALAS)	Cyclosporin (200 mg/kg p.o.)	Whole brain	16.2	22.5	1.4	*	Ejsing and Linnet, 2005
	10 mg/kg i.p	TCA	Rat (Wistar Hanover GALAS)	Cyclosporin (200 mg/kg i.p)	Whole brain	17.0	22.0	1.3	*	Ejsing and Linnet, 2005
	25 mg/kg i.p	TCA	Rat (Wistar Hanover GALAS)	Cyclosporin (200 mg/kg i.p)	Whole brain	23.0	27.0	1.2	ns	Ejsing and Linnet, 2005
	various (pooled)	TCA	Rat (Wistar Hanover GALAS)	Cyclosporin (200 mg/kg p.o.)	Whole brain	20.0	25.0	1.3	**	Ejsing and Linnet, 2005
→ ***E-OH-NT***	NOR 25 mg/kg p.o.	meta bolite	Rat (Wistar Hanover GALAS)	Cyclosporin (200 mg/kg p.o.)	Whole brain	1.2	2.0	1.7	ns	Ejsing and Linnet, 2005
	NOR 10 mg/kg i.p	meta bolite	Rat (Wistar Hanover GALAS)	Cyclosporin (200 mg/kg i.p)	Whole brain	~ 1.7	~1.9	1.1	ns	Ejsing and Linnet, 2005
	NOR 25 mg/kg i.p	meta bolite	Rat (Wistar Hanover GALAS)	Cyclosporin (200 mg/kg i.p)	Whole brain	~ 0.9	~ 1.1	1.2	ns	Ejsing and Linnet, 2005
	NOR various (pooled)	meta bolite	Rat (Wistar Hanover GALAS)	Cyclosporin (200 mg/kg p.o.)	Whole brain	1.3	1.6	1.3	ns	Ejsing and Linnet, 2005

Antidepressant	Dose	Class	Species (strain)	P-gp inhibitor	Brain Region	Brain/Serum Conc.			Sig	Ref
						Control	P-gp I	P-gp I / Control		
Risperidone[1]	1 mg/kg i.p.	n/a (APD)	Mouse (CF1)	Sertraline (SSRI; 10 µg/g i.p.)	Whole brain	1.6	2.3	1.4	**	Wang *et al.*, 2006a
→ ***9-OH-Risp***	n/a	meta bolite	Mouse (CF1)	Sertraline (SSRI; 10 µg/g i.p.)	Whole brain	0.9	2.5	2.8	**	Wang *et al.*, 2006a

P-gp I = group treated with P-gp inhibitor
BS: brain stem; FC: frontal cortex; HIPPO: hippocampus; HYPO: hypothalamus
[1] Risperidone is an antipsychotic drug (APD) known to be a P-gp substrate. This study demonstrates P-gp inhibition by sertraline *in vivo*.
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = non-significant
→ *denotes a metabolite*

1.5.6. Pharmacogenetic studies in humans

Given that preclinical findings have identified many antidepressants as P-gp substrates (Sections 1.5.4 and 1.5.5), and that medicated depressed patients have been reported to have increased P-gp activity (de Klerk *et al.*, 2009) (discussed in Section 1.4.6), it is plausible that P-gp efflux of antidepressants may be clinically relevant in resistance to the treatment of depression (as outlined in relation to other brain disorders in Section 1.3.5).

Co-administration of a P-gp inhibitor, itraconazole, has been reported to increase the bioavailability of paroxetine in humans (Yasui-Furukori *et al.*, 2007), while fluvoxamine and citalopram plasma pharmacokinetics were found to be dependent on polymorphisms in the *ABCB1* gene (Fukui *et al.*, 2007; Nikisch *et al.*, 2008), indicating that these antidepressants are P-gp substrates in humans.

As outlined in Section 1.3.4, the functional significance of any of the many SNPs in the *ABCB1* gene is unclear and remains controversial. Nonetheless, similar links have also been reported between *ABCB1* genotype and the frequency of side effects following antidepressant treatment (Roberts *et al.*, 2002; de Klerk *et al.*, 2013).

Moreover, several studies have reported associations between *ABCB1* SNPs and response to treatment with antidepressants, including paroxetine, citalopram and escitalopram (Gex-Fabry *et al.*, 2008; Kato *et al.*, 2008; Nikisch *et al.*, 2008; Sarginson *et al.*, 2010; Lin *et al.*, 2011; Singh *et al.*, 2012). In contrast, no links were found between these *ABCB1* SNPs and treatment response for amitriptyline, fluoxetine or nortriptyline (Roberts *et al.*, 2002; Laika *et al.*, 2006). Importantly, there has been little consensus regarding the involvement of particular SNPs in pharmacogenetic studies reporting positive results. Moreover, reported associations for paroxetine and citalopram have not been replicated in subsequent studies (Mihaljevic Peles *et al.*, 2008; Peters *et al.*, 2008), thereby raising concerns over the validity of these observations.

The majority of clinical studies in this area have focused on a small number of *ABCB1* SNPs, mostly at the 3435 and 2677 sites. An early study found postural hypotension to be significantly more common following treatment with nortriptyline in patients carrying the C3435T SNP (Roberts *et al.*, 2002). It was hypothesized that this may have been due to a relative increase in the accumulation of nortriptyline, or its metabolites, in the brain due to reduced P-gp function. Clinical response to nortriptyline (or fluoxetine) was not associated with the C3435T SNP in this study, however (Roberts *et al.*, 2002). In contrast, paroxetine treatment-response has been reported to have a significant association with *ABCB1* genotype (Gex-Fabry *et al.*, 2008; Kato *et al.*, 2008). However, the SNPs that were linked to paroxetine treatment-response differed between these two studies. Kato and colleagues reported a significant positive association between the G2677T/A SNP and paroxetine treatment-response. Furthermore, a link between the *ABCB1* haplotype combination 3435C–2677G–1236T and a poor therapeutic response to paroxetine was identified (Kato *et al.*, 2008). These associations did not reach significance in the other above-mentioned study, however, in which a link was found between a SNP at the 61 position in the *ABCB1* gene and the therapeutic response to paroxetine (Gex-Fabry *et al.*, 2008). In addition, a later study failed to replicate the findings of Kato and colleagues (Mihaljevic Peles *et al.*, 2008). It should, however, be noted that these studies were undertaken in patient cohorts from different ethnic backgrounds (Japanese vs. Caucasians)

Furthermore, the associations reported between *ABCB1* SNPs and paroxetine treatment response have not been observed with other antidepressants. In fact, Nikisch and colleagues (2008) reported directly conflicting findings with regard to citalopram, with the 2677T genotype being significantly more common in non-responders to citalopram treatment. This correlated with significantly lower plasma and cerebrospinal fluid citalopram concentrations in patients with the 2677T genotype relative to carriers of the wild-type allele, while no difference was observed in the same study for the C3435T polymorphism (Nikisch *et al.*, 2008). However, the small number of subjects in this pilot study (n=15) is a limitation. In addition, no significant correlation was found between the G2677T/A

polymorphism and therapeutic response or side effect severity in patients treated with amitriptyline (Laika *et al.*, 2006).

Another study failed to find any correlation between the C3435T polymorphism and the frequency of antidepressant-induced mania in bipolar patients, treated with various serotonergic antidepressants (De Luca *et al.*, 2003). However, the wide range of antidepressants involved in the study, some of which have been reported to be P-gp substrates *in vivo* (paroxetine, venlafaxine, fluoxetine etc.) and others which have not (sertraline, nefazodone, moclobemide), and the focus on an independent polymorphism, as opposed to its role as part of a haplotype, are major confounding factors (De Luca *et al.*, 2003).

The often contradictory literature surrounding the role of genetic differences in *ABCB1* in antidepressant response is complicated by several factors, not least the focus on individual SNPs, as opposed to haplotypes, in the majority of studies and the lack of clarity regarding the functional impact of these SNPs. In addition, in many cases it has not been clear if the antidepressants in question are transported substrates of human P-gp.

Rather than focusing on individual, previously described SNPs, Uhr and co-workers undertook a comprehensive fine-mapping of the *ABCB1* gene to identify variants which may be associated with antidepressant response (Uhr *et al.*, 2008). Moreover, in this study, antidepressants were classified as P-gp substrates (namely venlafaxine, citalopram, paroxetine and amitriptyline) or non-P-gp substrates (mirtazapine), albeit based on findings from studies in P-gp knockout mice rather than data pertaining to human P-gp.

In this study, no association was found between antidepressant treatment response and the G2677T or C3435T SNPs (Uhr *et al.*, 2008). This finding in relation to the G2677T or C3435T SNPs was subsequently replicated for citalopram, following analysis of data from the large Sequenced Treatment Alternatives to Relieve Depression (STAR*D) sample of depressed patients (Peters *et al.*, 2008).

In contrast, two key intronic *ABCB1* SNPs, rs2032583 and rs2235015, were identified as strong predictors of remission when patients were treated with antidepressants that were P-gp substrates (Uhr *et al.*, 2008). Patients carrying the rare alleles of these intronic SNPs and receiving treatment with P-gp substrate antidepressants had a 7.7-fold higher chance to remit after 5 weeks than carriers of the other alleles (Uhr *et al.*, 2008). This association was not due to differences in dosing or plasma levels, thus suggesting a role for P-gp function at the BBB. Moreover, no such association was determined in patients treated with mirtazapine, which is not a P-gp substrate (Uhr *et al.*, 2008), thus supporting the P-gp-related hypothesis. A later study partially replicated these findings for paroxetine (P-gp substrate) and mirtazapine (non-substrate) in geriatric depression, finding rs2032583, but not rs2235015, to be associated with response to paroxetine treatment (Sarginson *et al.*, 2010). However, in their retrospective analysis of data from the large STAR*D clinical trial population, Peters and colleagues failed to replicate the findings of Uhr and co-workers with regard to an association between certain *ABCB1* SNPs and citalopram response (Peters *et al.*, 2008). While the original focus of their study involved investigating potential links between three common *ABCB1* variants (C1236T, G2677T/A, and C3435T) and citalopram response, two *ABCB1* SNPs reported to be in tight linkage disequilibrium with rs2032583 in the first haplotype block reported to be significant by Uhr and colleagues were also genotyped. Neither of these SNPs were associated with citalopram response in the STAR*D population (Peters *et al.*, 2008; Peters *et al.*, 2009). However, it must be stressed that the authors did not have genotype data on the actual SNPs reported to be at play by Uhr and co-workers.

In contrast to Uhr and colleagues' findings in relation to P-gp substrate antidepressants, no association was found between duloxetine response and rs2032583 (Perlis *et al.*, 2010). Bearing in mind that there are no published *in vitro* or *in vivo* studies indicating that duloxetine is a transported P-gp substrate, this finding does not necessarily contradict the previous study. Further research is required to establish if duloxetine is a transported substrate of P-gp to facilitate informed interpretation of this study's finding in relation to rs2032583.

Significant links were also found between escitalopram response and uncommon *ABCB1* SNPs in Taiwanese depressed patients (Lin *et al.*, 2011). However, the loci associated with remission in this patient cohort were different to those identified by Uhr and co-workers. Moreover, a recent study has reported that the C3435T SNP predicts the dose of escitalopram required to achieve remission, with significantly higher doses required in carriers of the C allele (Singh *et al.*, 2012). Although citalopram, a racemic mixture of R- and S-enantiomers, has been identified as a P-gp substrate *in vivo*, no study has yet investigated the effect of P-gp on the brain distribution of escitalopram, the pharmacologically active S-enantiomer of citalopram. This is a key consideration given that P-gp is known to exhibit enantioselectivity for several drugs (de Lagerie *et al.*, 2004; Wang *et al.*, 2004b; Sakugawa *et al.*, 2009; Akamine *et al.*, 2012). Thus, further studies are needed to ascertain if escitalopram is a transported substrate of P-gp.

In summary, while several studies have highlighted potential pharmacogenetic interactions between *ABCB1* and antidepressant treatment, there has been little consistency regarding the involvement of particular SNPs and most studies have not been successfully replicated. However, recent findings regarding the rs2032583 SNP offer much promise. Further studies investigating interactions between this SNP and response to antidepressants which are transported substrates of human P-gp are now warranted. However, it should be noted that efflux transporters other than P-gp, such as breast cancer resistance protein (BCRP) and the multidrug resistance-associated proteins (MRPs), are also expressed at the BBB. The role of these transporters in the brain distribution and treatment response to antidepressants has been under-investigated, especially in light of a study linking response to citalopram treatment with a SNP in *ABCC1*, the gene encoding MRP1 (Lee *et al.*, 2010). Future studies should be conducted to address this issue.

1.5.7. Stress, the HPA axis, depression and P-gp

Stress is thought to play a major role in the development of depression (Gold and Chrousos, 2002). The hypothalamic-pituitary-adrenal (HPA) axis controls the physiological response to stress through the secretion of corticosteroid hormones

such as cortisol, the main glucocorticoid in humans. As discussed in Section 1.4.1, dysregulation of the HPA axis has been implicated in the pathogenesis of depression (Holsboer, 2000; Julio-Pieper and Dinan, 2010). The HPA axis is regulated by negative feedback inhibition, whereby secreted corticosteroid hormones act on the hypothalamus and pituitary gland to suppress further HPA axis activation by reducing corticotrophin-releasing factor (CRF) and adrenocorticotropic hormone (ACTH) secretion, respectively.

Hyperactivity of the HPA axis is one of the most consistent biological findings in depressed patients (Pariante and Lightman, 2008). Depressives often have elevated plasma cortisol (Halbreich *et al.*, 1985) and ACTH (Carroll *et al.*, 2007) levels, and fail to respond to the dexamethasone suppression test (Carroll, 1982a; Carroll, 1982b), whereby administration of the synthetic glucocorticoid dexamethasone should suppress the secretion of cortisol. This is indicative of central HPA axis overdrive, with compromised negative feedback inhibition at the hypothalamic level of the HPA axis, in depressed patients.

The effect of P-gp on the brain penetration of glucocorticoids is unclear and somewhat controversial. Several early studies reported that many important endogenous and synthetic glucocorticoids are P-gp-substrates (Ueda *et al.*, 1992; Vankalken *et al.*, 1993; Schinkel *et al.*, 1995; Meijer *et al.*, 1998; Karssen *et al.*, 2001; Karssen *et al.*, 2002; Uhr *et al.*, 2002). However, subsequent evidence, from experiments involving double knockout *abcb1ab*$^{(-/-)}$ mice, suggests that corticosterone (the main glucocorticoid in rodents) and cortisol (the main glucocorticoid in humans) are not P-gp substrates at the BBB *in vivo* (Mason *et al.*, 2008; Mason *et al.*, 2010), despite initial reports to the contrary (Uhr *et al.*, 2002). Moreover, recent studies have revealed that the brain accumulation of the glucocorticoids cortisol, corticosterone and dexamethasone is greater in single knockout *abcb1a*$^{(-/-)}$ mice than wild-types, thereby further confusing the issue (Mason *et al.*, 2012).

Regardless of this controversy, it has been reported that double P-gp knockout *abcb1ab*$^{(-/-)}$ mice have consistently lower plasma ACTH and corticosterone levels

than control animals, thus indicating that P-gp may play a key role in the regulation of the HPA axis, and that absence of P-gp results in sustained suppression of the HPA axis (Muller *et al.*, 2003; Yau *et al.*, 2007). Furthermore, treatment with the tricyclic antidepressant desipramine reduced both basal and activated HPA axis activity in wild-type comparator mice, while the same treatment only reduced an activated HPA axis in *abcb1ab*$^{(-/-)}$ mice (Yau *et al.*, 2007). This difference observed between P-gp knockout and wild-type mice further indicates that there may be a P-gp-dependent mechanism at play in the regulation of the HPA axis. However, the uncertainty regarding the involvement of P-gp in the BBB transport of endogenous glucocorticoids makes it difficult to explain this phenomenon. The original hypothesis, which proposed that an absence of P-gp at the BBB resulted in a suppression of the HPA by increased negative feedback inhibition axis due to enhanced corticosterone entry into the brain (Muller *et al.*, 2003; Yau *et al.*, 2007), is contingent on corticosterone being a transported substrate of P-gp at the BBB. Further investigations are therefore needed to fully explain these observations.

While hyperactivity of the HPA axis is associated with depression, successful antidepressant therapy results in normalization of the HPA axis (Herr *et al.*, 2003; Kunzel *et al.*, 2003). Antidepressant-mediated enhancement of glucocorticoid receptor function has been demonstrated *in vitro* (Pariante *et al.*, 1997; Pariante *et al.*, 2001; Herr *et al.*, 2003; Pariante *et al.*, 2003a; Pariante *et al.*, 2003b). Interestingly, this effect has been observed following antidepressant co-incubation with certain glucocorticoids, such as dexamethasone and cortisol, but was not evident with others, such as corticosterone (Pariante *et al.*, 1997). Given the lack of clarity regarding the role of P-gp in glucocorticoid transport, it is tempting to speculate that these differences are due to differential transport of glucocorticoids by P-gp. Further experiments are required to substantiate or disprove this hypothesis. Interestingly, co-incubation with the P-gp-inhibitor verapamil attenuated or reduced the glucocorticoid-enhancing effect of the antidepressants clomipramine and fluoxetine (Pariante *et al.*, 2001; Pariante *et al.*, 2003a; Pariante *et al.*, 2003b), thus indicating that antidepressant-mediated P-gp-inhibition may be the underlying mechanism at play. Therefore, it has been suggested that

antidepressant drugs may increase the access of cortisol to the brain by inhibition of P-gp at the BBB, thereby enhancing glucocorticoid-mediated negative feedback on the HPA-axis, and that this may contribute to the clinical mechanism of action of antidepressants (Figure 1.12) (Pariante *et al.*, 2004).

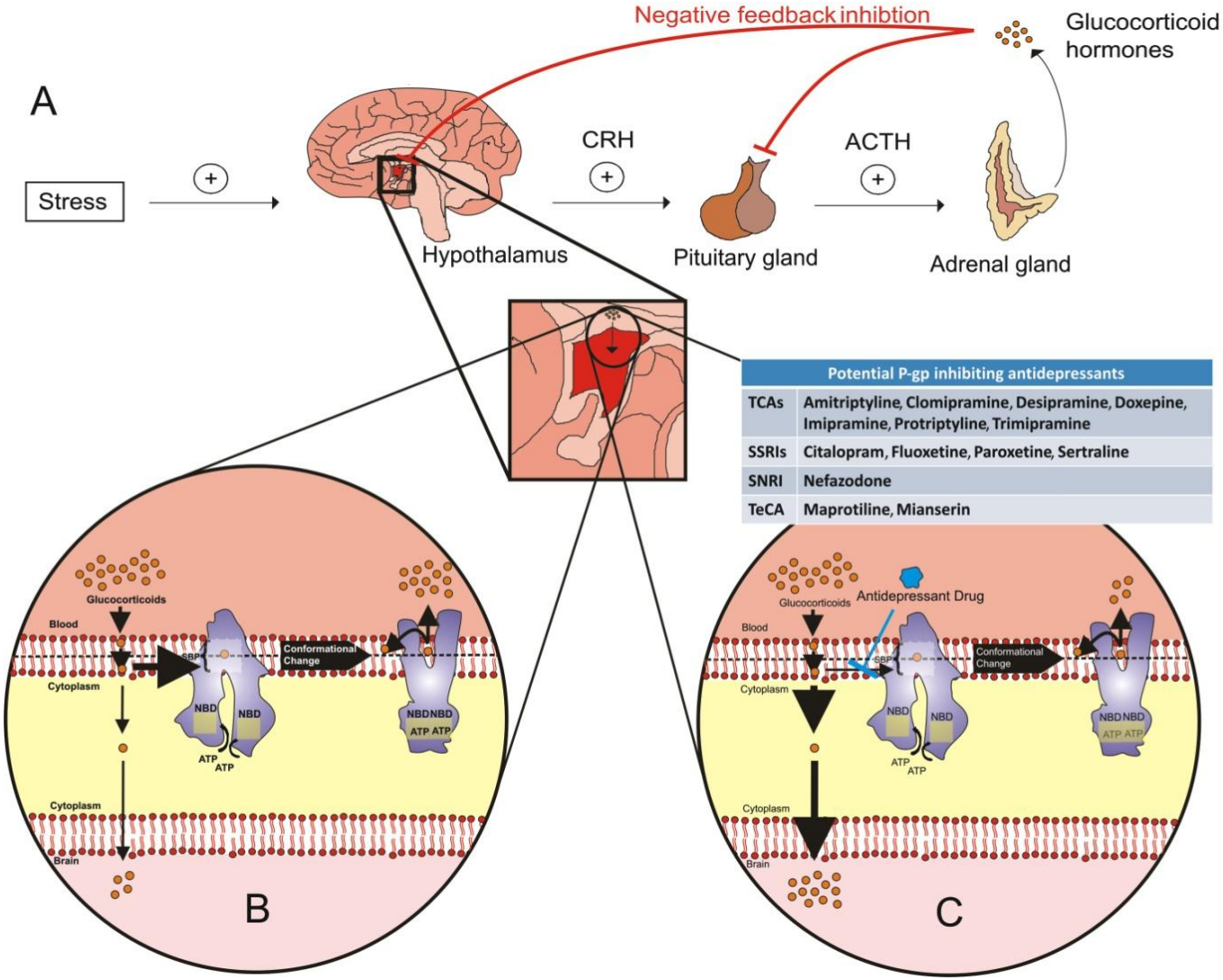
This theory has, however, been challenged (Weber *et al.*, 2005; Weber *et al.*, 2006). Firstly, it has been questioned if P-gp inhibition by antidepressants could be clinically relevant given that it is only seen at concentrations above therapeutically relevant plasma levels (Weber *et al.*, 2005). Furthermore, it seems that P-gp is present in excess in brain capillaries (Miller, 2010), which may influence the concentration of P-gp inhibitor needed to meaningfully increase substrate transport across a membrane (Kannan *et al.*, 2009). As a result, for antidepressants to exert clinically significant P-gp inhibition at the BBB, concentrations several times higher than their IC_{50} would need to be achieved (Kalvass and Pollack, 2007). Therefore, it remains unclear if P-gp inhibitory concentrations of antidepressants are reached at the BBB in clinical use.

In the second study challenging the relevance of P-gp inhibition to HPA axis normalising ability of antidepressants (Weber *et al.*, 2006), a potentially sub-effective dose of amitriptyline was used (Yau *et al.*, 2007). Moreover, it involved the comparison of corticosterone levels in the brain and plasma, and it has subsequently been reported that corticosterone may not a P-gp substrate *in vivo* (Mason *et al.*, 2008), thus undermining conclusions drawn from the study. Nonetheless, despite ample *in vitro* data demonstrating P-gp inhibition and P-gp-mediated enhancement of glucocorticoid function by antidepressants, there is a paucity of *in vivo* data to support the hypothesis that antidepressants inhibit P-gp, resulting in enhanced glucocorticoid entry into the brain. In fact, the most recent publication from the Pariante group (the original proposers of this hypothesis) in relation to this question has failed to demonstrate that either acute or chronic administration of the antidepressant drug desipramine results in enhanced brain levels of glucocorticoids in an *in situ* mouse model (Mason *et al.*, 2011). This study, in conjunction with another reporting enhanced P-gp function following chronic

antidepressant treatment (de Klerk *et al.*, 2010) undermines the hypothesis, and therefore it seems unlikely that antidepressant mediated inhibition of P-gp contributes to the clinical mechanism of action of antidepressant drugs.

In summary, the theory suggesting that P-gp inhibition at the BBB by some antidepressants may contribute to their mechanism of action is certainly interesting, but there is an increasing body of evidence undermining the hypothesis. The single study demonstrating P-gp inhibition by an antidepressant *in vivo* (Wang *et al.*, 2006a) has not yet been replicated. Furthermore, it has yet to be proven that P-gp-inhibitory concentrations of antidepressants are reached at the BBB in the clinical setting. It is also necessary to clarify the role of P-gp in the brain accumulation of glucocorticoids, and the underlying mechanism behind the reduced corticosterone levels observed in P-gp knockout mice relative to controls. In addition, P-gp function has been shown to be increased in medicated depressed patients, chronically treated with antidepressants, relative to healthy controls (de Klerk *et al.*, 2009). This would seem to contradict the theory that antidepressant treatment inhibits P-gp function at the BBB. However, the antidepressants used by the patients in this study were not listed. Therefore, it is not possible to determine if patients were treated with antidepressant medications that are thought to inhibit P-gp. Most importantly, a relatively recent study failed to demonstrate that administration of the antidepressant desipramine resulted in P-gp inhibition leading to enhanced glucocorticoid entry into the brain, thereby questioning the validity of the original hypothesis (Mason *et al.*, 2011). Therefore, on balance, evidence to support the theory that P-gp inhibition by antidepressants at the BBB contributes to their mechanism of action is controversial and not supported by the most recent *in vivo* studies. More research in this area is needed to prove whether such negative effects generalise to other antidepressants.

Figure 1.12: Potential interaction between regulation of the HPA axis and antidepressants as P-gp inhibitors* (O'Brien *et al.*, 2012b).
A. Stress activates the HPA axis by triggering the release of CRH from the hypothalamus, which in turn acts on the anterior pituitary gland to secrete ACTH. ACTH stimulates the release of glucocorticoid hormones from the adrenal cortex. These glucocorticoid hormones, including cortisol, the main glucocorticoid in humans, inhibit the release of CRH and ACTH from the hypothalamus and pituitary gland respectively, thus exerting negative feedback control on the system. However, control of the HPA axis is often dysregulated in depressed patients, who may exhibit elevated levels of circulating cortisol and ACTH due to hyperactivity of the HPA axis.
B. Many glucocorticoids are P-gp substrates. Therefore, in depressed patients, P-gp may limit the entry of glucocorticoids into the brain at the BBB, thus resulting in reduced negative feedback inhibition of the HPA axis.
C. Successful treatment of depression results in normalisation the HPA axis, and many antidepressant drugs have been shown to enhance GR activity in vitro - an effect attenuated or reduced by co-incubation with a P-gp inhibitor. Therefore, it has been hypothesized that part of the mechanism of action of these antidepressant drugs may be to facilitate the access of glucocorticoids to the hypothalamus by inhibiting P-gp, thereby restoring central negative feedback control of the HPA axis.
** It should be noted that this hypothesis is undermined by the most recent in vivo study with the antidepressant desipramine (Mason et al., 2011)*


A
Stress
Hypothalamus
CRH
Pituitary gland
ACTH
Adrenal gland
Glucocorticoid hormones
Negative feedback inhibtion
Potential P-gp inhibiting antidepressants
TCAs
Amitriptyline, Clomipramine, Desipramine, Doxepine, Imipramine, Protriptyline, Trimipramine
SSRIs
Citalopram, Fluoxetine, Paroxetine, Sertraline
SNRI
Nefazodone
TeCA
Maprotiline, Mianserin
Glucocorticoids
Blood
Cytoplasm
Conformational Change
NBD
ATP
Brain
B
Antidepressant Drug
C

1.5.8. Inhibition of P-gp as a putative antidepressant augmentation strategy?

While several preclinical studies have robustly identified various antidepressants as transported P-gp substrates in rodents, these findings have generally not been supported by investigations using *in vitro* bidirectional transport assays. From a clinical perspective, interactions between antidepressant drugs and P-gp could potentially lead to altered dose-to-plasma relationships and/or frequency of side effects with altered P-gp function. In particular, P-gp-mediated efflux of antidepressants at the BBB would limit the access of these drugs to their site of action within the brain, therefore potentially contributing to the high rate of treatment failure (Figure 1.13).

Further preclinical studies are required to more fully elucidate the role of P-gp in determining the brain levels and effect of antidepressant drugs *in vivo*. To date, no antidepressant has been robustly identified as a transported P-gp substrate both *in vitro* and *in vivo*. In particular, it is difficult to draw definitive conclusions from the available *in vitro* studies investigating if antidepressants are transported substrates of human P-gp. Thus, there is a major need for additional research in this regard. In addition, there is a paucity of studies demonstrating that brain concentrations of an antidepressant can be augmented by pharmacological inhibition of P-gp. Indeed, the only antidepressant drugs for which this has been reported are imipramine and nortriptyline, both of which are seldom used TCA antidepressants. Given that previous studies reported substantially smaller increases in the brain levels of nortriptyline following P-gp inhibition than similar experiments involving P-gp knockout mice (Doran *et al.*, 2005; Ejsing and Linnet, 2005), it is important to investigate the potential for modulation of P-gp as a strategy to enhance brain delivery. Moreover, the pharmacodynamic impact of increasing brain concentrations of P-gp substrate antidepressants has not been adequately investigated to date.

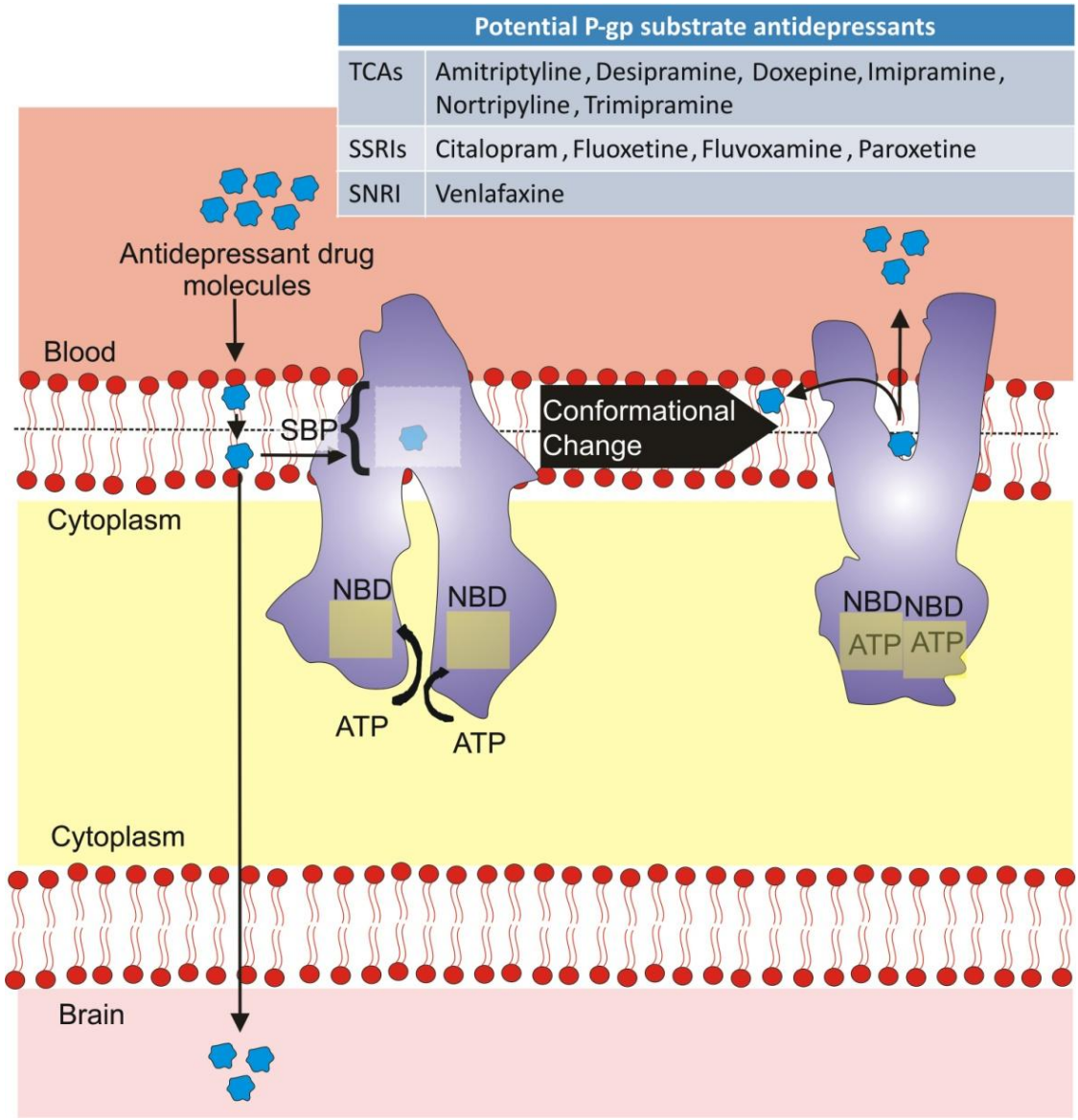


Figure 1.13: Antidepressants as transported substrates of P-gp at the BBB (O'Brien *et al.*, 2012b). *Based on numerous in vitro and in vivo studies, it is thought that many antidepressant drugs may be transported P-gp substrates at the BBB, thereby limiting their brain distribution. Therefore, therapeutically effective brain concentrations might not be achieved in some cases, which in turn may play a role in treatment resistant depression.*

Many studies have reported associations between SNPs in *ABCB1* and antidepressant treatment response. However, there appears to be little consensus among the reported results, with numerous publications claiming antithetical findings. Nonetheless, the report of Uhr and colleagues in 2008 is extremely promising (Uhr *et al.*, 2008), especially considering that some of their findings were

subsequently replicated (Sarginson *et al.*, 2010); although it must also be noted that others have failed to replicate certain aspects of these findings (Peters *et al.*, 2008). Thus, the importance of the putative role of *ABCB1* variants in the clinical efficacy of antidepressants remains controversial, and well-powered, prospective clinical trials are needed to definitively resolve this issue (Peters *et al.*, 2009).

Until such time as these trials have taken place, it remains plausible that P-gp inhibitors may augment the effects of certain antidepressants, particularly in treatment-resistant situations. Indeed, anecdotal evidence indicates a beneficial effect of adjunctive verapamil treatment in severely depressed patients who are refractory to SSRI treatment (Clarke *et al.*, 2009). Clinical trials, exercising caution to avoid potential unwanted side effects due to putative pharmacokinetic or pharmacodynamic interactions, investigating this possibility may now be warranted.

As outlined in Section 1.4.2, one common approach used in the treatment of TRD is the augmentation of a failed antidepressant therapy with another agent, such as the antipsychotics aripiprazole, olanzapine, quetiapine or risperidone (Philip *et al.*, 2010). Interestingly, some of these antipsychotics inhibit P-gp *in vitro* (Wang *et al.*, 2006b; Zhu *et al.*, 2007; Feng *et al.*, 2008); it is tempting to speculate that this property may contribute to their therapeutic efficacy as adjunctive treatments. Indeed, co-administration of risperidone with sertraline has been shown to result in a trend for increased sertraline concentrations in the brain of CF1 mice *in vivo* (Wang *et al.*, 2006a). Therefore, one could hypothesize that the mechanistic explanation for this augmentation strategy involves the inhibition of P-gp by the antipsychotic drug, leading to increased central antidepressant concentrations. However, further studies (both *in vitro* and *in vivo*) are needed to establish if there is any merit to this hypothesis.

In conclusion, recent studies suggest that a complex, and as yet not fully understood, relationship exists between antidepressants and drug efflux pumps, such as P-gp, at the BBB, and that this relationship may impact on the therapeutic efficacy of antidepressant treatment. TRD represents a major challenge in the

treatment of depression, and there is a huge unmet medical need for novel approaches to overcome this challenge. Advances in pharmacogenomics, neuroimaging and animal studies, coupled with more sophisticated *in vitro* assay technology, are sure to shed light on the interaction between antidepressants and P-gp in the coming years, thus potentially resulting in new strategies for adjunctive therapies for TRD.

1.6. Aims of the thesis

This thesis aims to further investigate interactions between P-gp and antidepressants at the BBB by addressing some key outstanding questions. In particular, the role played by P-gp in the brain distribution of certain antidepressants, such as escitalopram, has yet to be investigated. Moreover, it remains unclear if antidepressants which have been identified as transported P-gp substrates in rodents are also transported substrates of human P-gp. Most importantly in the context of P-gp inhibition as a putative strategy to augment antidepressant treatment, the effect of blocking P-gp on an antidepressant's activity in a relevant animal model remains to be elucidated.

1.6.1. Aim 1: Establish an integrated intracerebral microdialysis approach to determine the effect of P-gp inhibition on antidepressant levels in the brain

The intracerebral microdialysis approach offers several advantages as a means to assess the impact of P-gp on the brain distribution of drug compounds, as outlined in Section 1.2.5.4. Furthermore, by combining intracerebral microdialysis with peripheral jugular vein and carotid artery catheterizations, it would be possible to administer the drug of interest intravenously, thereby removing differences in drug absorption as a confounding factor, as well as taking parallel serial blood samples, thereby facilitating simultaneous monitoring of brain and plasma drug levels. Thus, it would be possible to determine the effect of administration of a P-gp inhibitor on the blood-brain barrier transport of antidepressant drugs in conscious freely moving animals over a period of time. We aimed to develop such a model and to apply it to different antidepressants to investigate the role of P-gp in their BBB transport (*Chapters 2 and 4*).

1.6.2. Aim 2: Establish and employ an *in vitro* model to identify antidepressants which are transported substrates of human P-gp

Due to reports of species differences in P-gp activity and specificity (Yamazaki *et al.*, 2001; Katoh *et al.*, 2006; Baltes *et al.*, 2007; Syvanen *et al.*, 2009), and indeed differences in the identification of P-gp substrates between single and double P-gp knockout mice, the clinical relevance of preclinical studies demonstrating that P-gp restricts the brain concentrations of various antidepressants in rodents is unclear. The establishment and consistent, rational application of an *in vitro* bidirectional transport model involving cells expressing human P-gp was therefore a priority, given the paucity and inconsistency of the available data pertaining to the transport of antidepressants by human P-gp. We aimed to establish such a model, and to use it to screen a number of antidepressant in relation to their status as transported P-gp substrates (*Chapters 3 and 4*).

1.6.3. Aim 3: Investigate the impact of P-gp inhibition on the pharmacodynamic activity of antidepressants which are P-gp substrates

While numerous studies have demonstrated that P-gp inhibition or P-gp knockout results in increased brain levels of various antidepressants, it has not yet been determined if enhancing brain levels of antidepressants in this way results in an augmentation in their antidepressant-like activity. We aimed to address this question, using appropriate animal models to investigate behavioural responses to antidepressant treatment, as outlined in Section 1.4.5 (*Chapters 4, 5 and 6*). Furthermore, we aimed to adapt our microdialysis model to investigate the impact of P-gp inhibition on antidepressant-induced increases in extracellular serotonin concentrations (*Chapter 5*).

1.6.4. Aim 4: Determine the effect of chronic treatment with a P-gp inhibitor and an antidepressant on the brain distribution and behavioural and molecular responses to antidepressant treatment

To date, studies investigating the effect of P-gp inhibition on the brain distribution of antidepressants have exclusively involved acute treatments. Given that antidepressants are prescribed on a chronic basis in clinical practice and considering that the expression of P-gp, as well as other ABC transporters, can be up-regulated in response to certain xenobiotics (Miller, 2010), it is essential to determine if P-gp inhibition will result in enhanced brain levels of a P-gp substrate antidepressant after chronic treatment. Moreover, there is typically a therapeutic delay of several weeks in the onset of action of antidepressants clinically (Krishnan and Nestler, 2008). Therefore, the impact of P-gp inhibition on responses to chronic antidepressant treatment is a key question (*Chapter 6*).

Chapter 2:
Inhibition of P-glycoprotein Enhances Transport of the Antidepressant Imipramine across the Blood-Brain Barrier: Microdialysis Studies in the Conscious Freely Moving Rat

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Abstract

Background: Recent studies indicate that efflux of antidepressants by the multidrug resistance transporter P-glycoprotein (P-gp) at the blood-brain barrier (BBB) may contribute to treatment resistant depression (TRD) by limiting intracerebral antidepressant concentrations. In addition, clinical experience shows that adjunctive treatment with the P-gp inhibitor verapamil may improve the clinical outcome in TRD. Therefore, the present study aimed to investigate the impact of P-gp inhibition on the BBB transport of the tricyclic antidepressant imipramine and its active metabolite desipramine.

Methods: Intracerebral microdialysis was used to monitor brain levels of imipramine and desipramine following intravenous imipramine administration, with or without pre-treatment with one of the P-gp inhibitors verapamil or cyclosporin A (CsA). Plasma drug levels were also determined at regular intervals.

Results: Pre-treatment with either verapamil or CsA resulted in significant increases in imipramine concentrations in the microdialysis samples, without altering imipramine plasma pharmacokinetics. Furthermore, pre-treatment with verapamil, but not CsA, led to a significant elevation in plasma and brain levels of desipramine.

Conclusions: The present study demonstrates that P-gp inhibition can enhance intracerebral imipramine concentrations, thus supporting the hypothesis that P-gp restricts brain levels of certain antidepressants, including imipramine. These findings may help to explain reports of a beneficial response to adjunctive therapy with verapamil in TRD.

2.1. Introduction

Recent pre-clinical studies indicate that several antidepressant drugs may be substrates of the multidrug resistance transporter P-glycoprotein (P-gp) at the blood-brain barrier (BBB) (O'Brien *et al.*, 2012b). P-gp, encoded by the *ABCB1* gene in humans, is expressed at multiple sites within the body, including the luminal membrane of the brain capillary endothelial cells which form the BBB (Cordoncardo *et al.*, 1989). Drug efflux by P-gp at the BBB represents a major obstacle in the delivery of pharmacological compounds which are P-gp substrates to the brain, and has been linked to treatment failure in various brain disorders, such as epilepsy and brain cancer (Siddiqui *et al.*, 2003; Pauwels *et al.*, 2007).

Several functional single nucleotide polymorphisms (SNPs) in the *ABCB1* gene have been identified which impact on P-gp expression and/or function (Cascorbi, 2006). These functional SNPs may therefore have an impact on the ability of P-gp substrate drugs to reach the brain. Emerging clinical evidence indicates that the response rate to treatment with antidepressants, particularly those which have been shown to be P-gp substrates at the BBB *in vivo*, may be associated with *ABCB1* genotype (Uhr *et al.*, 2008; Sarginson *et al.*, 2010; Lin *et al.*, 2011). This suggests that P-gp-mediated efflux may contribute to the high prevalence of treatment resistant depression (TRD) by limiting antidepressant concentrations at their intracerebral site of action (O'Brien *et al.*, 2012b). In addition, preliminary clinical studies have suggested that co-administration of the P-gp inhibitor verapamil may be beneficial in TRD (Clarke *et al.*, 2009). However, despite numerous *in vivo* studies demonstrating enhanced brain levels of several antidepressants in P-gp knockout mice compared to wild-type controls, the relative contribution of P-gp-mediated efflux and passive permeability on the BBB transport of these antidepressant drugs has yet to be definitively determined, and the significance of putative P-gp efflux to the BBB transport of antidepressants remains contentious (O'Brien *et al.*, 2012b). Not all antidepressants are P-gp substrates, however. Mirtazapine is one such example (Uhr *et al.*, 2003; Uhr *et al.*, 2008), and interestingly clinical response to mirtazapine is not associated with *ABCB1* genotype (Uhr *et al.*, 2008). Furthermore,

the complexity of the relationship between antidepressants and P-gp should be noted, as well as the difficulties in extrapolating results from one experimental context to another (O'Brien *et al.*, 2012b). This point is illustrated by work from the Pariante group, where *in vitro* studies led to the proposal that activity of P-gp at the BBB may itself be a target of antidepressant action (Pariante *et al.*, 2003b; Pariante *et al.*, 2004), whereas recent *in vivo* results appear to contradict this hypothesis (Mason *et al.*, 2011).

While several studies have investigated the distribution of antidepressants into the brain in P-gp knockout mice relative to wild-type controls, there is a paucity of research investigating the impact of pharmacological P-gp inhibition on the ability of antidepressants to penetrate the BBB, with only three such studies published to date to our knowledge (Ejsing and Linnet, 2005; Ejsing *et al.*, 2006; Clarke *et al.*, 2009). We have previously shown that pre-treatment with verapamil leads to enhanced brain-to-serum ratios of the tricyclic antidepressant imipramine and its active metabolite, desipramine, in certain brain regions (Clarke *et al.*, 2009). However, *in vitro* transport studies using different P-gp expressing cell-lines have yielded conflicting findings regarding the P-gp substrate status of imipramine (Mahar Doan *et al.*, 2002; Faassen *et al.*, 2003). Therefore, it remains unclear whether P-gp limits the ability of imipramine to penetrate the BBB. These previous studies in normal animals have only investigated brain-to-serum ratios at a single (terminal) time-point post-antidepressant administration, and therefore have not elucidated the time-dependent impact of P-gp inhibition on the distribution of these antidepressants into the brain.

The present study utilises an integrated intracerebral microdialysis technique to investigate the impact of pre-treatment with one of two distinct P-gp inhibitors, verapamil or cyclosporin A (CsA), on free imipramine and desipramine concentrations in brain extracellular fluid (ECF) over time in wild-type Sprague Dawley rats. Intracerebral microdialysis represents a useful investigational tool which can be used to assess drug permeability across the BBB as a function of time,

particularly when applied to the study of drug transporters in the central nervous system (CNS) (Sawchuk and Elmquist, 2000).

2.2. Methods

2.2.1. Drugs and chemicals

High performance liquid chromatography (HPLC) grade acetonitrile, potassium dihydrogen phosphate and orthophosphoric acid (OPA) were obtained from Fisher Scientific (Ireland). Heparin sodium solution (Wockhardt UK Ltd, UK) and CsA (Sandimmun; Novartis Pharmaceuticals UK Ltd, UK) were purchased from Uniphar Group (Ireland). Imipramine, desipramine, trimipramine and verapamil were obtained from Sigma-Aldrich as were all other chemicals unless otherwise stated.

2.2.2. Animals

Male Sprague Dawley rats (Harlan Laboratories, UK), weighing 255-290 g, were used in this study (total n = 18). Animals were group-housed 4-6 animals per cage and maintained on a 12 hour light/dark cycle (lights on at 08:00 h) with food and water ad libitum. Room temperature was controlled at 22 ± 1°C. All procedures were carried out in accordance with EU directive 89/609/EEC and approved by the Animal Experimentation & Ethics Committee of University College Cork.

2.2.3. Surgical procedures

Animals were anaesthetized prior to surgery with a ketamine/xylazine mixture (90/10 $mg.kg^{-1}$ i.p.), with maintenance of anaesthesia achieved by repeating 20-25% of the induction dose at 30-40 minute intervals, as required. Analgesia was provided by pre-operative administration of carprofen (3 $mg.kg^{-1}$ s.c.). Throughout surgical procedures, the body temperature of each rat was maintained using a heating pad.

2.2.3.1. Catheterization surgery

Two indwelling catheters were surgically implanted in each rat using standard surgical techniques: one in the carotid artery for collection of serial blood samples and one in the external right jugular vein to facilitate intravenous drug administration (Thrivikraman *et al.*, 2002; Huang *et al.*, 2006; Heiser, 2007). All catheters were pyrogen-free polyethylene tubing (Instech Laboratories, Plymouth Meeting, PA). The arterial and venous catheters consisted of BPE-T25 tubing

(0.018"ID x 0.036"OD) and BPE-T50 tubing (0.023"ID x 0.038"OD), respectively. The catheters were subcutaneously tunnelled to the back of the neck of the rat, where they were exteriorized and connected to three-way Discofix® stopcocks (B. Braun). To prevent clotting the catheters were filled with a heparinised saline solution (20 $IU.ml^{-1}$).

2.2.3.2. Microdialysis surgery

Immediately after completion of catheterization surgery, while still anaesthetized, rats were placed in a stereotaxic frame (Model 900 Small Animal Stereotaxic Instrument, David Kopf Instruments, Bilaney Consultants, St Julians, Sevenoaks, UK) with the skull flat. A small burr hole was made in the skull, centred 2.7 mm anterior and 0.7 mm lateral to bregma. The microdialysis probe was slowly lowered 5 mm from dura into the prefrontal cortex (PFC) (Paxinos and Watson, 1998) and secured with skull screws and dental acrylic. The inlet of the probe was connected to a fluid swivel (Instech Laboratories, Plymouth Meeting, PA) and the rats were single-housed in cylindrical plexiglass containers (Instech Laboratories, Plymouth Meeting, PA) filled with bedding and food pellets. The venous and arterial catheters were secured to the swivel in the cage to prevent the rats from interfering with them, while at the same time enabling free movement. Artificial cerebrospinal fluid (aCSF: 147 mM NaCl, 1.7 mM $CaCl_2$, 0.9 mM $MgCl_2$, and 4mM KCl) was continuously perfused through each microdialysis probe at a rate of 1.5 $\mu l.min^{-1}$ by a microlitre 'Pico Plus' syringe pump (Harvard Apparatus, Fircroft Way, Edenbridge, Kent UK) and the rats allowed to recover overnight prior to sampling on the following day during the optimal post-surgical period (de Lange *et al.*, 2000).

2.2.4. Dialysis probe construction and calibration

Vertical concentric microdialysis probes were constructed as described elsewhere (Page and Lucki, 2002). Briefly, a piece of fused silica (ID 75 ± 3 µm, OD 150 ± 6 µm; CM Scientific Ltd, UK) was inserted through PE10 tubing (Instech Laboratories, Plymouth Meeting, PA). A Spectra/Por® Micro-dialysis Hollow Fiber regenerated cellulose semipermeable membrane with a 13 kD molecular weight cut-off (Spectrum Laboratories, Inc.) was placed over the fused silica and into the PE10

tubing, and fixed in place using epoxy adhesive. The open end of the semipermeable membrane was sealed with a 0.5 mm epoxy plug, and the active area of the fibre, where diffusion takes place across the membrane, was limited to 3 mm in length by coating regions outside this range with epoxy adhesive. To determine the *in vitro* probe recovery rate, each probe was immersed in a well of aCSF containing a known concentration (100 $ng.ml^{-1}$) of imipramine and desipramine. The recovered concentration of imipramine and desipramine in the perfusate was expressed as a percentage of the known concentration in the well. Probes used in the *in vivo* studies had *in vitro* recovery rates between 6.8-12.2% for imipramine and 7.0-13.5% for desipramine. As imipramine is known to bind to plastic tubing (Friedl and Propping, 1984), it was not possible to calibrate the probes *in vivo* using standard techniques such as the no-net-flux or retrodialysis methods, due to imipramine binding to inlet (polyethylene) tubing in the probes (unpublished observations). As the diffusion properties of compounds in brain tissue are likely different from *in vitro* conditions, dialysate values were not corrected to account for the *in vitro* recovery rate of the probe. However, it was possible to directly compare the uncorrected dialysate concentrations between the groups as there was no statistical difference between *in vitro* probe recovery rates across the groups (Table 2.1), thus ensuring comparisons were valid as per numerous previous reports (Sato *et al.*, 1994; Evrard *et al.*, 1998; Page and Lucki, 2002; Page *et al.*, 2010).

2.2.5. Experimental design

Rats were separated into three groups: imipramine only (IMI only), imipramine plus verapamil (IMI+VER) and imipramine plus CsA (IMI+CsA; n=6 per group) (Figure 2.1). Imipramine (5 $mg.kg^{-1}$ i.v.) was administered to all rats via the jugular vein catheter, with or without pre-treatment with either verapamil or CsA, both of which are P-gp inhibitors. Rats in the IMI+VERAP group were pre-treated with verapamil (20 $mg.kg^{-1}$ i.p.) 90 minutes before imipramine administration. Rats in the IMI+CsA group were pre-treated with CsA (25 $mg.kg^{-1}$ i.v.) 30 minutes before imipramine administration. The doses, routes of administration and timing of P-gp inhibitor

administration used were based on those previously published in P-gp inhibition studies (Tsai *et al.*, 2002; Bart *et al.*, 2003; Syvanen *et al.*, 2006; Liow *et al.*, 2007; Clarke *et al.*, 2009). Microdialysis samples (dialysates) from the PFC were collected at 20 minute intervals for 1 hour before (blanks) and for 4 hours post-imipramine administration. Dialysates were stored at -80°C until analysed by HPLC. Blood samples were taken at 8 time-points, one before imipramine administration (blank) and one at 5, 15, 30, 60, 120, 180 and 240 minutes post-imipramine administration. Blood samples (~ 250 µl) were immediately centrifuged at 5000 rpm for 5 minutes, plasma taken and stored at -80°C until extraction for analysis by HPLC with electrochemical detection. At the conclusion of the experiment, rats were deeply anaesthetized using intravenous ketamine/xylazine mixture, and bromophenol blue dye was then infused through the probe to mark its location. The rats were subsequently decapitated while under anaesthesia, and the brains removed for histological verification of probe placement. Data were discarded if the probe placement was outside of the PFC.

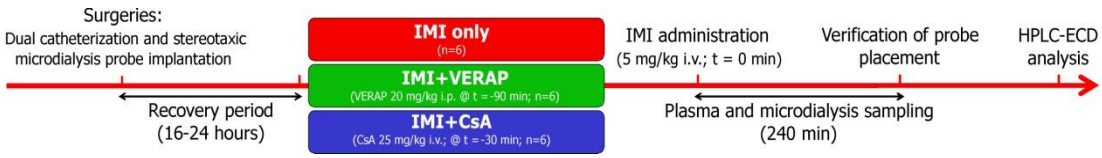

Figure 2.1: Schematic illustrating experimental design and timelines *(refer to text in Section 2.2.5 for full details).*

2.2.6. Plasma extraction

Imipramine and its active metabolite desipramine were extracted from plasma using a liquid-liquid extraction technique as described previously (Clarke *et al.*, 2009), with some modifications. Briefly, 98 µl of plasma was spiked with 2 µl of the internal standard, trimipramine, to yield a final concentration of 20 $ng.ml^{-1}$ trimipramine. To this trimipramine-spiked plasma, 1 ml of sodium hydroxide (2 M) and 3 ml of water were added. Extraction was carried out in 7.5 ml of 1.5% isoamyl alcohol in n-heptane by vortexing for 30 seconds, followed by agitation on a mechanical shaker for 15 min and then centrifugation at 5000 RPM for 15 min at

20°C. The upper solvent layer was transferred to a tube containing 200 µl of 25 mM OPA, vortexed for 30 seconds, then agitated on a mechanical shaker for 15 min followed by centrifugation at 5000 rpm for 15 min at room temperature. Twenty microlitres of the lower aqueous phase was injected onto the HPLC system for analysis.

2.2.7. HPLC analysis

2.2.7.1. HPLC equipment

The HPLC with electrochemical detection (HPLC-ECD) system consisted of a Shimadzu LC-20AD XR Prominence Pump, CBM-20A communication bus module, SIL-20AC XR Prominence Autosampler, CTO-20A Prominence Column oven (all supplied by Mason Technology, Cork, Ireland). System components were used in conjunction with Shimadzu LC solutions software (Mason Technology). The detector used was ESA Coulochem III with the 5041 Amperometric Cell (Supplied by ESA Analytical, Ltd., Brook Farm, Dorton, Aylesbury, Buckinghamshire, HP18 9NH England). All samples were injected onto a reversed phase Luna 3 µm C18(2) 150 x 2 mm column (Phenomenex), which was protected by Krudkatcher Ultra in-line filters (Phenomenex).

2.2.7.2. HPLC conditions

The HPLC-ECD method was adapted from previously described methods (Sato *et al.*, 1994; Frahnert *et al.*, 2003). Briefly, the mobile phase which was used on the HPLC system consisted of a mixture of potassium dihydrogen phosphate (25 mM, pH 7 with 4N NaOH) and HPLC grade acetonitrile (56:44). Mobile phase was filtered through Millipore 0.22 µm Durapore filters (Millipore, Ireland) and vacuum degassed prior to use. Compounds were eluted isocratically over a 30 min runtime at a flow rate of 0.4 $ml.min^{-1}$ after a 20 µl injection. The column was maintained at a temperature of 30^{o}C and samples/standards were kept at 8^{o}C in the cooled autoinjector prior to analysis. The glassy carbon working electrode combined with a platinum reference electrode (ESA) was operated at a potential of 600 mV and a range of 50 nA.

2.2.7.3. Analyte identification and quantification

Imipramine, desipramine, verapamil and trimipramine (internal standard) were identified by their characteristic retention times as determined by standard injections which were run at regular intervals during sample analysis. For the extracted plasma samples, analyte:internal standard peak height ratios were measured and compared with standard injections, and results were expressed as nanograms of analyte per ml of plasma. As no extraction procedure was necessary for microdialysis samples, analyte peak heights from undiluted samples were compared directly with standard injections and expressed as nanograms of analyte per ml of dialysate. The limit of detection (S/N ratio of 2) was 0.22 ng/ml and 0.28 ng/ml for desipramine and imipramine respectively, while the limit of quantification (S/N ratio of 5) was 0.56 ng/ml for desipramine and 0.71 ng/ml for imipramine. The co-efficient of variation (% CV) for the HPLC method was 2% for desipramine and 3% for imipramine (n=8). The accuracy of the technique was determined by carrying out the extraction procedure on plasma samples spiked with known concentrations of imipramine and desipramine followed by HPLC analysis. The mean (± SEM) concentration determined for extracted desipramine and impramine samples were 96 ± 1 % and 102 ± 1 % of the known spiked concentration respectively (n = 4).

2.2.8. Data analysis and statistical procedures

Pharmacokinetic (PK) parameters were calculated from the observed data by non-compartmental analysis. The area under the concentration-time curve (AUC) for imipramine and desipramine in plasma and dialysate samples was calculated using the linear trapezoidal method from the first to the last measured plasma concentration in all cases. The elimination rate constant (k_{el}) for imipramine in plasma was calculated by log-linear regression of the last four plasma concentration time-points. The apparent terminal elimination half-life ($t_{1/2}$) of imipramine in plasma was obtained from the k_{el}. Imipramine clearance from plasma (Cl) after i.v. bolus dose administration was calculated by dividing the i.v. dose by the AUC. All statistical analyses were carried out using standard commercial software (PASW Statistics, version 17.0.2; SPSS, Inc., Chicago, IL). Plasma and dialysate concentration

versus time profiles were compared using one-way repeated measures ANOVA. Statistical analyses of all other parameters were carried out using one-way ANOVA. Where a significant overall group effect was observed, the two-sided Dunnett's post-hoc test was used to elucidate differences between the IMI only group and the other two groups. All data are reported as mean ± SEM. The criterion for statistical significance was $p \leq 0.05$.

2.3. Results

2.3.1. Plasma imipramine and desipramine pharmacokinetics

There was no significant difference between the groups in terms of imipramine levels in plasma over time ($F(2, 15) = 0.696$, $p > 0.05$; Figure 2.2A). Furthermore, there was no significant difference in mean plasma imipramine AUC values between the groups ($F(2, 15) = 0.726$, $p > 0.05$; Table 2.1). Plasma pharmacokinetic parameters for imipramine are listed in Table 2.1. No statistically significant differences between the groups were observed in any of the plasma pharmacokinetic parameters.

Due to transient technical difficulties with baseline stabilisation pertaining to the electrochemical detection technique, it was not possible to determine plasma desipramine concentrations at certain time-points for 2 of the 18 animals in the study. As a result of these missing individual data-points, these animals could not be included in repeated measures analysis of plasma desipramine levels, thus reducing our group numbers to n= 5 for both the IMI only and IMI+CsA groups for the repeated measures ANOVA. Nonetheless, there was a significant overall group effect on plasma desipramine levels over time ($F(2, 13) = 53.311$, $p < 0.001$; Figure 2.2B), with significantly higher plasma desipramine concentrations in the IMI+VERAP group than the IMI only group at every time-point (Figure 2.2B). In addition, there was a significant group effect on mean plasma desipramine AUC values ($F(2, 15) = 40.689$, $p < 0.001$; Table 2.1), with post-hoc analysis revealing that the mean plasma desipramine AUC was significantly higher in the IMI+VERAP group than the IMI only group ($p < 0.001$), with no difference between the IMI only and IMI+CsA groups (Table 2.1).

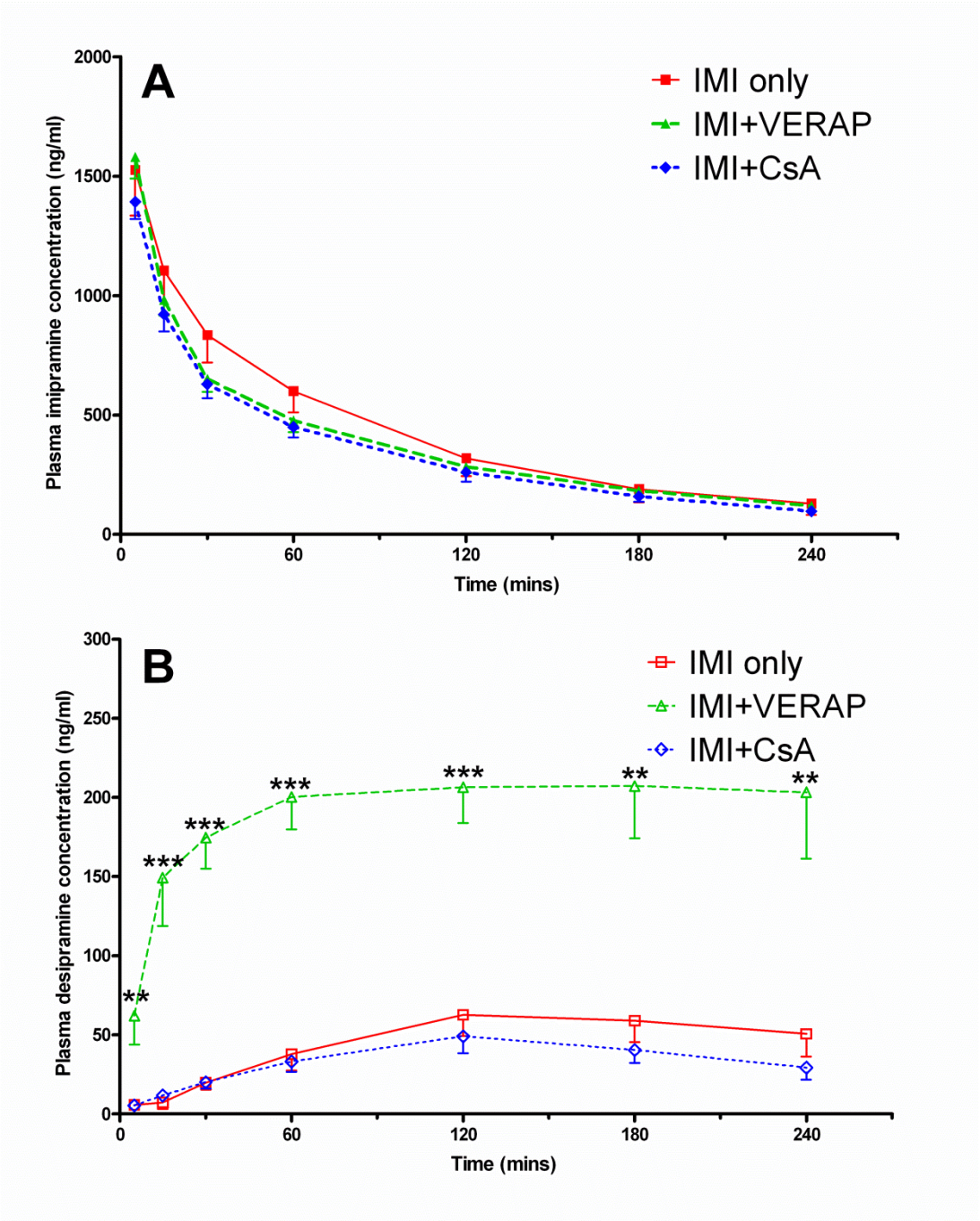

Figure 2.2: Plasma imipramine and desipramine profiles

*A. Imipramine concentrations in plasma as measured at different time points. There was no statistical difference in imipramine plasma profiles between the 3 groups. B. Desipramine concentrations in plasma as measured at different time points. There was a significant overall difference between the groups in terms of desipramine plasma profiles ($p < 0.001$). Desipramine levels were markedly increased in the IMI+VERAP group relative to the IMI only group at all time points (** $p < 0.01$; *** $p < 0.001$).*

Data are represented as mean ± SEM from 5-6 animals per group. Red square symbols with solid line represent IMI only group; green triangular symbols with dashed line represent IMI+VERAP group; blue diamond-shaped symbols with dotted line represent IMI+CsA group.

Table 2.1: Summary of key pharmacokinetic (PK) parameters and probe recovery values

		IMI only mean ± SEM	IMI+VERAP mean ± SEM	IMI+CsA mean ± SEM	Overall *p*-value
Plasma AUC ($ng.ml^{-1}.min$)	IMI	101 635 ± 18 370	88 002 ± 7876	81 041 ± 7350	0.500
	DMI	11 137 ± 2393	46 050 ± 4984 ***	7990 ± 1 527	< 0.001***
Imipramine plasma PK parameters	k_{el} (min^{-1} x 10^{-3})	9.5 ± 1.1	7.9 ± 0.6	8.8 ± 0.7	0.426
	$t_{1/2}$ (min)	79.8 ± 12.3	90.2 ± 7.0	81.5 ± 6.0	0.681
	Cl ($ml.min^{-1}$)	15.43 ± 2.13	16.05 ± 1.55	17.69 ± 2.21	0.713
***In vitro* probe recovery (%)**	IMI	8.23 ± 0.80	8.91 ± 0.48	8.92 ± 0.66	0.703
	DMI	8.62 ± 0.98	9.56 ± 0.68	9.18 ± 0.57	0.701
Dialysate AUC ($ng.ml^{-1}.min$)	IMI	1322 ± 98	1802 ± 144*	2108 ± 169**	0.004**
	DMI	n/a[1]	158.4 ± 14	n/a[1]	n/a
Ratio of imipramine dialysate AUC: plasma AUC		0.0149 ± 0.0024	0.0215 ± 0.0028	0.0274 ± 0.0038*	0.035*

AUC, area under the concentration curve; Cl, drug clearance from plasma; DMI, desipramine; IMI, imipramine k_{el}, elimination rate constant; $t_{1/2}$, half-life of drug in plasma.

* $p \leq 0.05$; ** $p < 0.01$; *** $p < 0.001$ (relative to IMI only group)

n/a = not available

[1] Below limit of quantification

2.3.2. Dialysate imipramine and desipramine pharmacokinetics

There was a significant overall group effect on dialysate imipramine concentrations over time ($F(2, 15) = 8.361$, $p < 0.01$; Figure 2.3A), with significantly greater dialysate imipramine concentrations observed in the IMI+CsA group than the IMI only group between the second and fifth samples inclusive (Figure 2.3A). In addition, there was a significant overall difference between the groups in terms of dialysate imipramine AUC values ($F(2, 15) = 7.533$, $p < 0.01$; Table 2.1). The increases in imipramine dialysate AUC values observed in the IMI+VERAP group (36% increase; $p = 0.05$) and IMI+CsA group (59% increase; $p < 0.01$) relative to the IMI only group were statistically significant. Desipramine levels in the dialysate samples proved to be below the limit of quantification in the IMI only and IMI+CsA groups. However, it was possible to quantify desipramine levels in the dialysate from rats in the IMI+VERAP group (Figure 2.3B; Table 2.1), thus suggesting that the levels were higher in this group although statistical analysis was precluded by the lack of quantifiable samples from the other two groups.

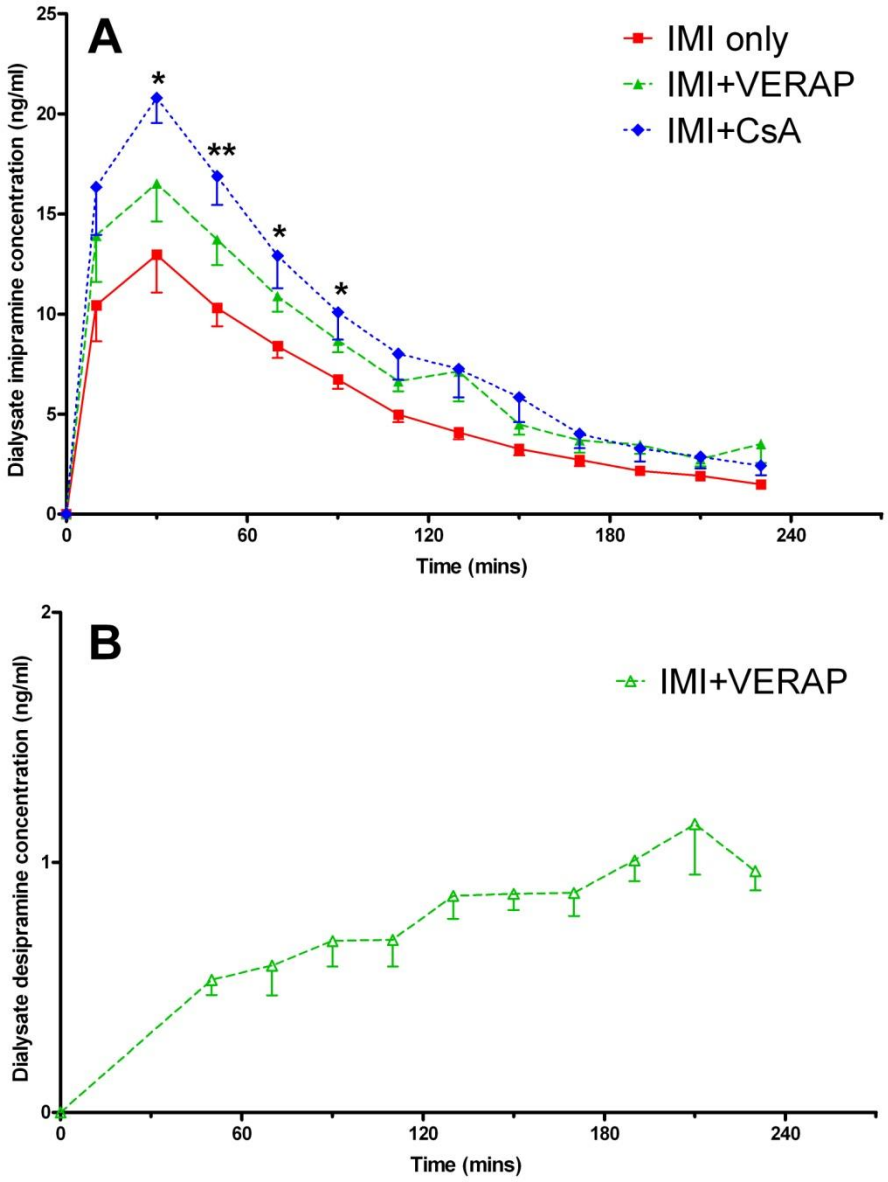

Figure 2.3: Dialysate imipramine and desipramine profiles

A. Imipramine concentrations in the dialysate samples as measured at different time intervals. Samples were continually collected and taken for analysis at 20 minute intervals. There was a significant overall group effect on dialysate imipramine concentrations over time ($p < 0.05$). Imipramine concentrations were significantly higher in the IMI+CsA group than the IMI only group between the second and fifth samples, inclusive ($p < 0.05$; ** $p < 0.01$). B. Dialysate desipramine concentrations as measured at different time intervals. Note: Desipramine concentrations in dialysate samples from the IMI only and IMI+CsA groups were below the limit of quantification. The first 5 time-points shown represent mean values of 3-5 animals per time-point. At these early time-points, values for the excluded animal(s) were below the limit of quantification and were therefore omitted.*

Mean ± SEM; n = 6. Red square symbols with solid line represent IMI only group; green triangular symbols with dashed line represent IMI+VERAP group; blue diamond-shaped symbols with dotted line represent IMI+CsA group.

2.3.3. Comparison of dialysate:plasma imipramine AUC ratios

The dialysate:plasma imipramine AUC ratio gives an indication of BBB transport as it accounts for any variations in plasma imipramine levels which might explain observed differences in dialysate concentration. There was a significant overall group effect on the ratio of dialysate:plasma imipramine AUC ($F(2, 15) = 4.210$, $p < 0.05$; Table 2.1). Post-hoc analysis revealed that the 84% increase observed in the IMI+CsA group relative to IMI only group was statistically significant ($p < 0.05$), while the 44% increase observed in the IMI+VERAP group relative to the IMI only group was not statistically significant (Figure 2.4).

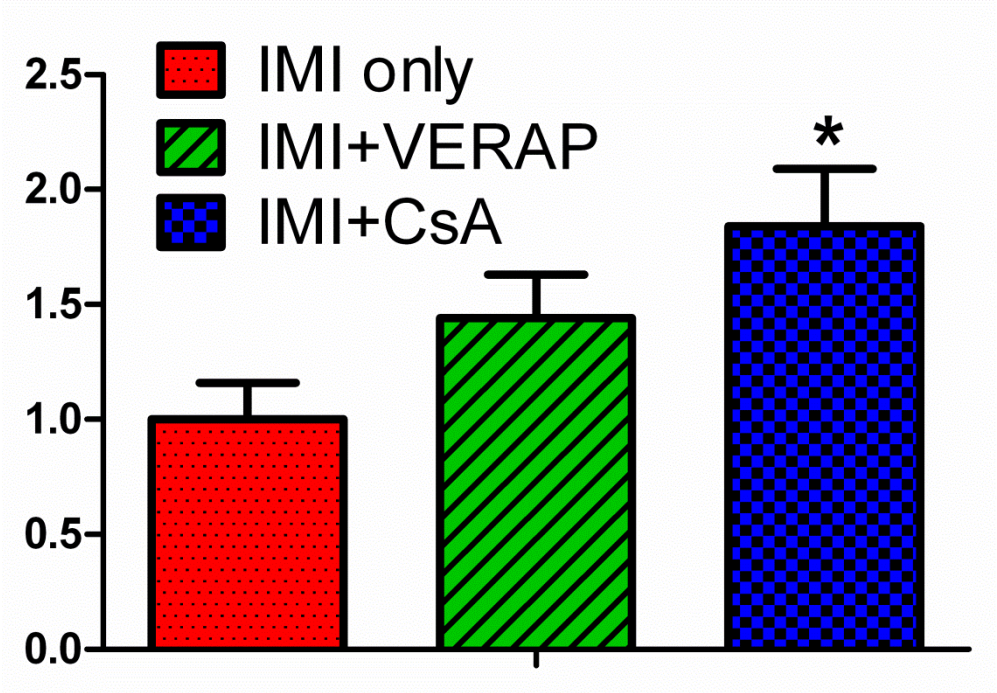

Figure 2.4: Comparison of dialysate:plasma imipramine AUC ratio
Dialysate:plasma imipramine area under the curve (AUC) ratio for each group, normalised to IMI only group. There was a significant overall group effect ($p < 0.05$), with post-hoc analysis showing that the 84% increase evident in the IMI+CsA group is statistically significant ($p < 0.05$). The 44% increase observed in the IMI+VERAP group did not reach statistical significance.*
Data are represented as mean ± SEM from 6 animals per group. Horizontally dotted red bar represents IMI only group; diagonally striped green bar represents IMI+VERAP group; black and blue checked bar represents IMI+CsA group.

2.4. Discussion

Despite some conflicting reports in the literature, much recent evidence highlights a potential link between therapeutic response to antidepressants which have been shown to be P-gp substrates and functional SNPs in the *ABCB1* gene which encodes P-gp in humans (Gex-Fabry *et al.*, 2008; Kato *et al.*, 2008; Nikisch *et al.*, 2008; Uhr *et al.*, 2008; Sarginson *et al.*, 2010; Lin *et al.*, 2011). The present study demonstrates that pre-treatment with either verapamil or CsA, both of which are well-established P-gp inhibitors, significantly increases levels of the tricyclic antidepressant imipramine in brain ECF without affecting imipramine levels in plasma. This offers the intriguing possibility of adjunctive treatment with P-gp inhibitors to enhance the BBB penetrating ability of certain antidepressant therapies, which may be particularly relevant to TRD.

Furthermore, the potential to lower the dose of the antidepressant required to achieve the same clinical effect when administered in combination with a P-gp inhibitor may help to negate some of the problematic peripheral side-effects associated with antidepressant therapy. Both verapamil and CsA are first generation P-gp inhibitors with other pharmacological activities aside from P-gp inhibition (Colabufo *et al.*, 2010). Verapamil is primarily used as a calcium channel blocker, while CsA is an immunosuppressive agent. Therefore, unwanted side-effects associated with other pharmacological actions would likely preclude the widespread use of these drugs to achieve adjunctive P-gp inhibition in the management of depression. More specific second and third generation P-gp inhibitors may represent viable alternatives due to reduced off-target effects. However, it is important to be cautious when extrapolating results from preclinical rodent studies to the clinical setting due to potential species differences in terms of P-gp substrate specificity (Yamazaki *et al.*, 2001; Baltes *et al.*, 2007).

The P-gp-inhibition-mediated increases in brain antidepressant levels occurred independently of any significant alteration in the plasma pharmacokinetics of imipramine. Clinical investigations have demonstrated that co-administration of verapamil increased imipramine bioavailability following oral administration in

healthy volunteers, and it was speculated that this effect was due to reduced oral clearance of imipramine (Hermann *et al.*, 1992). In the present study, pre-treatment with verapamil did not alter the plasma clearance of imipramine (Table 2.1). We previously reported a significant, but small, increase in serum imipramine concentration at a single (terminal) time-point following verapamil pre-treatment (Clarke *et al.*, 2009). This effect was not observed in the present study, possibly due to the different routes of imipramine administration employed in the two studies (i.p. previously *versus* i.v. in the present study) or the different methods of sampling (trunk blood collection following decapitation previously *versus* sampling from indwelling catheter in the present study).

The ratio of dialysate:plasma imipramine AUC for animals in the IMI+CsA group was 84% higher than for animals in the IMI only group. This observed difference was statistically significant, which strongly suggests enhanced imipramine transport across the BBB in CsA-treated animals. Although dialysate imipramine AUC was significantly higher in the IMI+VERAP group than the IMI only group, the 44% difference in the dialysate:plasma imipramine AUC ratio observed between these groups did not reach statistical significance, likely due to the increased variability observed when plasma concentrations were taken into account. As both CsA and verapamil are well characterised as P-gp inhibitors (Baumert and Hilgeroth, 2009), these data corroborate our previous findings (Clarke *et al.*, 2009) and further support the hypothesis that P-gp limits the ability of certain antidepressants, including imipramine, to cross the BBB (O'Brien *et al.*, 2012b). Moreover, it is worth noting that the greater increase in brain imipramine levels observed in the CsA pre-treated group compared with verapamil pre-treated animals is in agreement with CsA being a more potent P-gp inhibitor than verapamil (Hsiao *et al.*, 2008).

To our knowledge, the present study is the first to employ the microdialysis technique to investigate the effect of P-gp inhibition on the ability of antidepressants to cross the BBB and enter the brain. The microdialysis approach offers several advantages over traditional methods which involve the analysis of drug concentrations in brain homogenate samples (de Lange *et al.*, 2000;

Hammarlund-Udenaes, 2010). Firstly, samples obtained using the microdialysis technique only contain drug which has penetrated the BBB, whereas samples obtained from brain homogenates are confounded by the presence of a network of blood capillaries throughout the brain. Secondly, microdialysis measures only free drug molecules in the brain ECF, whereas the traditional brain homogenate method does not typically differentiate between pharmacologically inactive bound and active unbound drug. Thirdly, using the microdialysis technique it is possible to track fluctuations in brain drug concentrations in individual animals over time with or without P-gp inhibition, thus providing temporal resolution which is not possible with homogenate-based approaches. In addition, microdialysis enables us to investigate the impact of P-gp inhibition on drug concentrations in a specific brain region of interest, such as the PFC. While region-specific analysis can be carried out using brain homogenate techniques (Clarke *et al.*, 2009), previous studies have almost exclusively focused on whole brain antidepressant drug concentrations rather than investigating region-specific effects. This is a major limitation of such studies considering that there are differences in P-gp expression and function between different anatomical regions in the brain (Kwan *et al.*, 2003; Clarke *et al.*, 2009). Therefore, the use of whole brain analysis previously may have obscured important region-specific differences in drug concentrations.

When comparing dialysate concentrations which have not been corrected to account for *in vivo* probe recovery in microdialysis studies investigating the impact of P-gp efflux on the BBB transport of a drug, it is important to consider that altering drug clearance by P-gp inhibition will likely have an impact on probe recovery (Sawchuk and Elmquist, 2000). However, using mathematical modelling, it has been predicted that *in vivo* probe recovery would be lower in situations where efflux processes have been reduced, such as P-gp inhibition (Bungay *et al.*, 1990). Moreover, this has proven to be the case experimentally (de Lange *et al.*, 1998; Xie *et al.*, 1999). Therefore, it is likely that if it were possible to correct our dialysate concentrations for *in vivo* recovery, and thereby calculate actual brain ECF imipramine concentrations, we would in fact see an even more exaggerated

increase in brain imipramine levels in the verapamil and CsA pre-treated animals relative to the untreated controls.

Given the different methodology employed in the present study, it is difficult to compare the magnitude of the increases in brain imipramine concentrations reported here with those reported previously in other studies which have investigated the impact of P-gp inhibition on the BBB transport of antidepressants. Moreover, it will be of interest to assess how these findings will relate to human patients treated chronically with antidepressants.

Interestingly, verapamil pre-treatment markedly enhanced the concentrations of desipramine, an active metabolite of imipramine and an antidepressant in its own right, in plasma. The mean AUC for desipramine in plasma in the IMI+VERAP group was over 4-fold greater than the corresponding value in the IMI only group and almost 6 times greater than the desipramine plasma AUC in the CsA pre-treated group. We previously reported a 1.3-fold, non-significant increase in serum desipramine levels in verapamil pre-treated rats relative to controls (Clarke *et al.*, 2009). The greater difference observed in the present study is likely due to differences in the experimental protocol (i.e. dosing regimen and sampling methods) as discussed previously. The mechanism underlying this verapamil-mediated increase in plasma desipramine concentrations is unclear at present. However, it seems unlikely that it is mediated by P-gp inhibition, as there was no increase in plasma desipramine levels in CsA pre-treated animals. In addition to its primary pharmacological role as a calcium channel antagonist and its ability to inhibit P-gp, verapamil interacts with various other transporters, receptors and metabolic enzymes (Auguet *et al.*, 1986; Cole *et al.*, 2000; Polasek *et al.*, 2004; Wang *et al.*, 2004d). Moreover, verapamil has been shown to alter the pharmacokinetics of several drugs (Backman *et al.*, 1994; Kantola *et al.*, 1998; Fleishaker *et al.*, 2000; Kovarik *et al.*, 2005). Thus, it seems likely that the increase in plasma desipramine levels might be mediated via one of these alternative interactions rather than by inhibition of P-gp.

In particular, verapamil has been described as an inhibitor of cytochrome P450 enzymes (Wang *et al.*, 2004d), and these enzymes play the predominant role in imipramine and desipramine metabolism in humans (Lemoine *et al.*, 1993). While co-administration of verapamil did not alter imipramine plasma clearance (Table 2.1), one could speculate that the increase in plasma desipramine levels observed in the IMI+VERAP group may be due to verapamil-mediated inhibition of desipramine metabolism. In addition, it is worth noting that a functional cytochrome P450 monooxygenase system exists in the brain (Ravindranath, 1998). Therefore, putative inhibition of these enzymes by verapamil could impact on drug levels in the brain.

The increased desipramine levels in plasma observed in the IMI+VERAP group were reflected in the brain, where desipramine levels in microdialysis samples could only be reliably quantified in animals pre-treated with verapamil, thus indicating higher brain levels in these animals. Whether this increase in brain desipramine concentrations plays a role in the putative therapeutic benefit of verapamil augmentation in TRD, as anecdotally reported (Clarke *et al.*, 2009), remains to be clarified. However, it should be noted that a human study in healthy volunteers found that verapamil co-administration had no effect on desipramine concentrations in plasma following oral administration of imipramine (Hermann *et al.*, 1992). Therefore, the increase in desipramine levels following verapamil pre-treatment in rats treated with i.v. imipramine observed here might not be relevant in humans receiving oral imipramine treatment. As it was not possible to quantify desipramine concentrations in dialysate samples from the IMI only or IMI+CsA groups, it was not possible to determine whether pre-treatment with either P-gp inhibitor had any impact on the BBB transport of desipramine.

In conclusion, the present study clearly demonstrates that pre-treatment with either verapamil or CsA, both of which are P-gp inhibitors, results in increased brain levels of the antidepressant imipramine in wild-type rats. This shows that P-gp efflux limits the ability of imipramine to cross the BBB. These findings support the increasing body of clinical evidence which indicates that P-gp mediated restriction

of the ability of certain antidepressants to reach their site of action within the brain could potentially be a contributing factor to the high prevalence of TRD. In light of these findings, it is tempting to speculate that adjunctive therapy with P-gp inhibitors may offer clinical benefits in the treatment of depression.

Chapter 3:

Human P-glycoprotein Differentially Affects Antidepressant Drug Transport: Relevance to Blood-Brain Barrier Permeability

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Abstract

The pharmacological concept that inhibition of the drug efflux pump P-glycoprotein (P-gp) enhances brain distribution of the antidepressant imipramine in the rat has recently been demonstrated. To determine if these findings are relevant to humans, the present studies investigate if imipramine is a transported substrate of human P-gp. Furthermore, additional experiments were carried out to determine if findings in relation to imipramine and human P-gp would apply to other antidepressants from a range of different classes. To this end, bidirectional transport experiments were carried out in the *ABCB1*-transfected MDCKII-MDR1 cell line. Transported substrates of human P-gp are subjected to net efflux in this system, exhibiting a transport ratio (TR) ≥ 1.5, and directional efflux is attenuated by co-incubation of a P-gp inhibitor. Imipramine was identified as a transported substrate of human P-gp (TR = 1.68, attenuated by P-gp inhibition). However, the antidepressants amitriptyline, duloxetine, fluoxetine and mirtazapine were not transported substrates of human P-gp (TR ≤ 1.16 in all cases). The present findings offer insight into the role of P-gp in the distribution of antidepressants, revealing that rodent findings pertaining to imipramine may translate to humans. Moreover, the present results highlight that other antidepressants may not be transported substrates of human P-gp.

3.1. Introduction

Overcoming treatment resistant depression represents a major challenge in psychiatry, with up to 50-60% of depressed patients not achieving adequate response following antidepressant treatment (Fava, 2003). Increasing preclinical evidence suggests that the multidrug resistance transporter P-glycoprotein (P-gp), expressed at the blood-brain barrier (BBB), plays an important role in the brain distribution of several, but not all, antidepressants (O'Brien *et al.*, 2012b). These findings may be of considerable clinical importance given that drug efflux by P-gp can prevent therapeutic brain concentrations of centrally acting drugs from being achieved, thereby resulting in treatment failure (Loscher and Potschka, 2005b).

Indeed, several gene association studies have recently reported links between single nucleotide polymorphisms (SNPs) in *ABCB1*, the gene which encodes P-gp in humans, and treatment response to certain antidepressants (Gex-Fabry *et al.*, 2008; Kato *et al.*, 2008; Nikisch *et al.*, 2008; Uhr *et al.*, 2008; Sarginson *et al.*, 2010; Lin *et al.*, 2011; Singh *et al.*, 2012). These findings indicate that P-gp efflux at the BBB may contribute to the high failure rate of treatment with these antidepressants by preventing therapeutic brain levels from being achieved in some patients. However, similar pharmacogenetic associations have not been observed for other antidepressants (Roberts *et al.*, 2002; Laika *et al.*, 2006; Perlis *et al.*, 2010), indicating that P-gp efflux may not be clinically important for all antidepressant drugs. It is therefore important to determine if individual antidepressants are transported substrates of human P-gp to facilitate prediction of clinically relevant interactions with P-gp at the BBB.

P-gp is a 170-kDa membrane-bound broad-spectrum unidirectional efflux pump, also known as multidrug resistance protein 1 (MDR1). As a member of the adenosine triphosphate binding-cassette (ABC) superfamily of efflux transporters, P-gp uses ATP hydrolysis to power the active efflux of substrate molecules against a concentration gradient (Figure 3.1A). Owing to the diverse composition of its substrate-binding pocket, which contains distinct drug-binding sites consisting of different individual residues, P-gp exhibits remarkably broad polyspecificity and is

renowned for its substrate promiscuity (Aller *et al.*, 2009). P-gp is expressed at various sites in the body, including the apical membrane of the intestinal epithelial cells, the biliary canalicular membrane of hepatocytes and the luminal membrane of proximal tubular epithelial cells in the kidney (Marzolini *et al.*, 2004). In addition, P-gp is expressed at the luminal membrane of the endothelial cells in the cerebral vasculature, where it acts as a "gatekeeper at the BBB" (Schinkel, 1999).

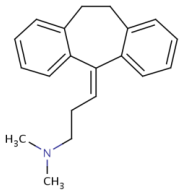
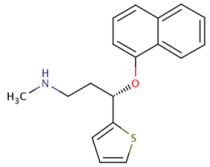
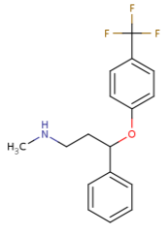
Despite the abundance of studies which have reported that P-gp restricts brain concentrations of various structurally unrelated antidepressants from different classes in rodents (Uhr *et al.*, 2000; Uhr and Grauer, 2003; Uhr *et al.*, 2003; Doran *et al.*, 2005; Ejsing and Linnet, 2005; Uhr *et al.*, 2008; Clarke *et al.*, 2009; Karlsson *et al.*, 2010; Bundgaard *et al.*, 2012; O'Brien *et al.*, 2012a; Karlsson *et al.*, 2013; O'Brien *et al.*, 2013b), there has been a paucity of studies which have investigated whether these antidepressants are also transported substrates of human P-gp. This is a major concern in light of well-documented interspecies differences in terms of P-gp substrate specificity and activity (Yamazaki *et al.*, 2001; Katoh *et al.*, 2006; Baltes *et al.*, 2007; Syvanen *et al.*, 2009). Of the few *in vitro* studies which have investigated interactions between human P-gp and antidepressants, many have used approaches which offer little information regarding drug transport, such as the ATPase assay, rather than adopting the gold-standard bidirectional transport approach (Balimane *et al.*, 2006; O'Brien *et al.*, 2012b). Furthermore, some of the bidirectional transport studies which have included antidepressants to date have reported conflicting results, which may be explained by differences in methodological approach and highlights well-documented problems associated with inter-lab variability (Balimane *et al.*, 2006; O'Brien *et al.*, 2012b). Therefore, it is imperative that additional research is carried out in this area to facilitate critical evaluation of the role P-gp plays in antidepressant distribution in humans.

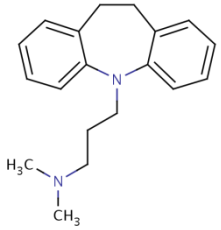
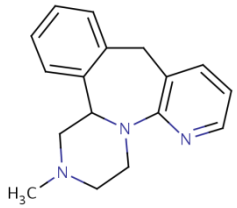
To this end, *in vitro* bidirectional transport studies were conducted in the *ABCB1*-transfected MDCKII-MDR1 cell line (Figure 3.1B) to build on our recent rodent data in relation to the tricyclic antidepressant imipramine (Clarke *et al.*, 2009; O'Brien *et al.*, 2012a, *Chapter 2*) by ascertaining if it is also a transported substrate of human

P-gp. Furthermore, we investigated if findings in relation to imipramine and human P-gp would apply to other structurally distinct antidepressants.

Five antidepressants were included in the present study, with representatives from each of the major antidepressant classes (Table 3.1). Despite their structural differences and different classes, these drugs have broadly similar physicochemical characteristics, with comparable molecular weights, lipophilicities and dissociation constants (Table 3.1). However, the selected antidepressants differ in terms of available information regarding their transport by P-gp from *in vivo* and *in vitro* studies (Table 3.1). We aimed to determine if previous *in vivo* findings in rodents would translate to studies involving human P-gp and to determine if previous *in vitro* findings would be replicated for the selected antidepressants, thereby enhancing our understanding of the role P-gp may play in the transport of these antidepressant drugs across the BBB in humans.

Table 3.1: Antidepressants investigated in the present studies, including their structures, antidepressant classes, physicochemical properties and results from previous *in vivo* and *in vitro* P-gp studies

Drug name and structure[a]	Antidepressant Class	Physicochemical properties			P-gp substrate?		Refs
		MW	Log P	pK_a	*In vivo*	Human P-gp	
 Amitriptyline	TCA	277.4[a]	4.9[a]	9.4[a]	Y?	Y/N	(Uhr *et al.*, 2000; Mahar Doan *et al.*, 2002; Faassen *et al.*, 2003; Grauer and Uhr, 2004; Uhr *et al.*, 2007)
 Duloxetine	SNRI	297.4[a]	4.0[a]	9.3[b]	n/a	n/a	n/a
 Fluoxetine	SSRI	309.3[a]	4.6[a]	8.7[b]	Y/N	N	(Uhr *et al.*, 2000; Mahar Doan *et al.*, 2002; Doran *et al.*, 2005; Feng *et al.*, 2008)

Drug name and structure[a]	Antidepressant Class	Physicochemical properties			P-gp substrate?		Refs
		MW	Log P	pK_a	*In vivo*	Human P-gp	
 Imipramine	TCA	280.4[a]	3.9[a]	9.4[a]	Y	Y/N	(Mahar Doan *et al.*, 2002; Faassen *et al.*, 2003; Clarke *et al.*, 2009; O'Brien *et al.*, 2012a)
 Mirtazapine	NaSSA	265.4[a]	2.9[a]	7.7[c]	N	n/a	(Uhr *et al.*, 2003; Uhr *et al.*, 2008)

Log P: partition co-efficient; MW: molecular weight; NaSSA: noradrenergic and specific serotonergic antidepressant; pK_a: acid dissociation constant; SNRI: serotonin and noradrenaline reuptake inhibitor; SSRI = selective serotonin reuptake inhibitor; TCA = tricyclic antidepressant
[a] www.drugbank.ca; [b] MSDS (ehs.lilly.com); [c] Remeron RD™ Monograph (www.remeronrd.ca)

3.2. Methods

3.2.1. Drugs and chemicals

High performance liquid chromatography (HPLC) grade acetonitrile, potassium dihydrogen phosphate and orthophosphoric acid (OPA) were obtained from Fisher Scientific (Ireland). Mirtazapine, duloxetine hydrochloride and fluoxetine hydrochloride were purchased from Discovery Fine Chemicals (Dorset, UK). Verapamil hydrochloride, imipramine hydrochloride and amitriptyline hydrochloride were obtained from Sigma-Aldrich (Ireland), as were all other chemicals and materials unless otherwise stated.

3.2.2. Cell-lines and materials

MDCKII-WT and MDCKII-MDR1 cells were obtained from the Piet Borst Laboratory (Netherlands Cancer Institute, Amsterdam, The Netherlands). Transport experiments were carried out using Costar 3414 Polycarbonate Membrane Transwell® Inserts (24 mm diameter, 3 µm pore size).

3.2.3. Cell culture

Cells were grown in 75 cm^2 culture flasks using Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. Cells were split twice weekly at a dilution of 1:12, and incubated at 37°C in a humidified 5% CO_2 and 95% air atmosphere.

3.2.4. Western blot analysis

Expression of P-gp was confirmed by western blot analysis once a month (Figure 3.1C). The western blot procedure was performed as described previously (Gosselin *et al.*, 2010). MDCKII-MDR1 and MDCKII-WT monolayers were grown as per transport experiments. Cells were washed with ice-cold phosphate-buffered saline, then lysed in lysis buffer (HEPES 20 mM; Np-40 (1%); DTT 0.5 mM; Na_3VO_4 0.1 mM; PMSF 0.1 mM; aprotinin 2 µg/ml; leupeptin 2 µg/ml; NaF 0.02 mM) for 15 min at 4°C. Cell lysate was centrifuged at 4°C for 10 min at 10 000 rpm. The supernatant was stored at -80°C for later analysis. Five micrograms of cell protein were loaded in

10-12% SDS-PAGE gel and run at 60-100 V for 120 min. After transfer onto a 0.2-µm nitrocellulose membrane and blocking with 5% skimmed milk and 0.1% Tween 20 in PBS, blots were probed overnight at 4°C with the C219 primary monoclonal P-gp antibody (1:100 dilution in 2% skimmed milk) (Enzo Life Sciences (UK) Ltd, Exeter, UK). Reprobing was conducted for 1 h at room temperature using a goat anti-mouse IgG-HRP conjugate, diluted 1:2000 (Jackson Immunoresearch Europe Ltd). Images were obtained using a luminescent image analyzer (LAS-3000; Fujifilm, Ireland). For the detection of β-actin, the membranes were incubated with Monoclonal Anti-β-Actin−Peroxidase antibody produced in mouse (1:15000). Immunoblots were quantified using ImageJ software.

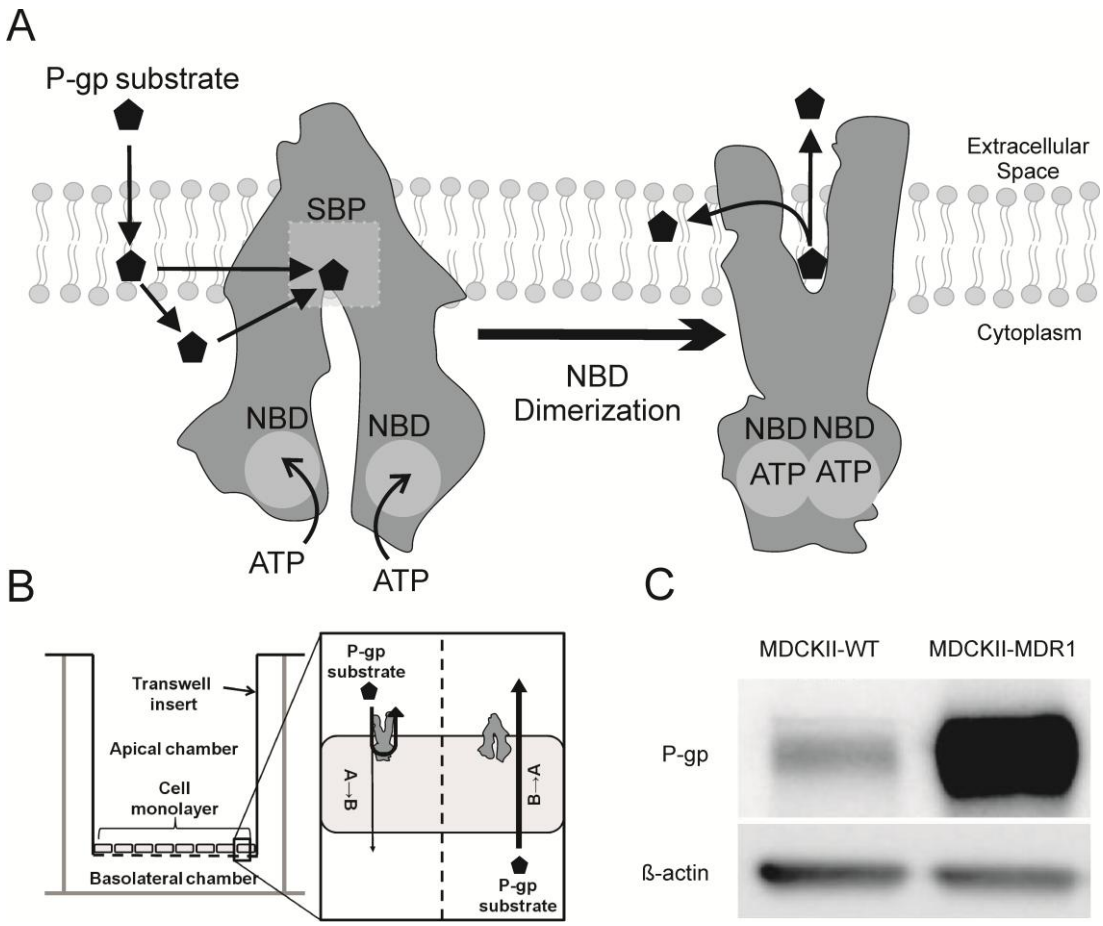


Figure 3.1*: A Mechanism of efflux by P-gp. P-gp substrates enter the P-gp substrate-binding pocket (SBP) via portals which are open to the cytoplasm and inner leaflet of the lipid bilayer. Binding of ATP to the nucleotide-binding domain (NBD), stimulated by interactions between the substrate and residues in the SBP, results in dimerization of the NBDs. This triggers a conformational change which sterically occludes transport of the substrate into the cytoplasm or the inner leaflet of the bilayer. Hence, when the substrate is released, it partitions into the outer leaflet of the bilayer or to the extracellular space, thereby preventing transport of the substrate across the lipid bilayer (Aller et al., 2009). B Schematic representation of the in vitro bidirectional transport model. A monolayer of MDCKII-MDR1 cells, cultured on a semi-permeable membrane at the base of a transwell insert, separates distinct apical and basolateral chambers. As human P-gp is expressed on the apical membrane of MDCKII-MDR1 cells only, it restricts the transport of P-gp substrates in the apical-to-basolateral (A→B) direction, but not in the basolateral-to-apical (B→A) direction. Therefore, transported substrates of human P-gp will exhibit lower permeability in the A→B than B→A direction, while non-substrates will have similar permeabilities in both directions. C Representative image of western blot, confirming greater expression of P-gp in MDCKII-MDR1 cells than MDCKII-WT cells.*

3.2.5. Transport experiments

The MDCKII-MDR1 cell line is stably transfected with *ABCB1* and expresses human P-gp on the apical membrane in a polarised fashion when cultured on an appropriate transwell support (Pastan *et al.*, 1988). Therefore, transported substrates of human P-gp will have lower permeability in the apical-to-basolateral (A→B) direction than in the basolateral-to-apical direction (B→A), due to the effect of P-gp efflux at the apical membrane, exhibiting a (B→A)/(A→B) transport ratio (TR) ≥ 2, while a TR between 1.5 and 2 is inconclusive (Polli *et al.*, 2001; Zhang *et al.*, 2006). As the MDCKII-MDR1 cell line also expresses endogenous canine kidney transporters, the TR in MDCKII-MDR1 cells can be compared to the TR in wild-type MDCKII-WT cells to yield a corrected transport ratio (cTR), which in theory isolates the effect of human P-gp. A cTR value of ≥ 1.5 indicates the test compound may be a transported substrate of human P-gp (Schwab *et al.*, 2003). However, transfection with *ABCB1* may alter the expression of endogenous transporters (Kuteykin-Teplyakov *et al.*, 2010). Therefore it is important to repeat transport experiments with inhibition of P-gp to confirm that P-gp is responsible for net efflux in MDCKII-MDR1 cells. In the present studies, where initial transport experiments indicated an antidepressant may be a transported P-gp substrate, experiments were repeated with co-incubation of the P-gp inhibitors verapamil (200 µM) and CsA (25 µM) as previously described (Tang *et al.*, 2002; Taub *et al.*, 2005). For transport experiments, cells (passage number: 8-30) were seeded at a density of $2x10^6$ cells per well. Cells were allowed to grow to confluency over 4 days with 1.5 ml of medium in the apical compartment and 2.6 ml of medium in the basolateral compartment. The medium was changed daily and trans-epithelial electrical resistance (TEER) measurements were taken each day using an Endohm meter (World Precision Instruments, Sarasota, FL) to monitor the development and integrity of the cell monolayer. On the days of experiments, the mean (± SD) TEER value for MDCKII-WT cells was 194 (± 43) $\Omega.cm^2$ and for MDCKII-MDR1 cells was 387 (± 36) $\Omega.cm^2$. Fresh cell culture medium was added two hours before the transport experiment. One hour later, cell culture medium was removed and monolayers were rinsed twice with transport buffer (Hank's Balanced Salt Solution

[HBSS] with 10mM HEPES). All transport experiments were carried out independently in three different transwells (n = 3). Transport experiments were conducted by adding the test drug (freshly prepared in transport buffer) to the donor chamber and measuring the appearance of drug at 30 minute intervals in the receiver chamber over a two hour incubation period at 37°C. Apical and basolateral chamber volumes were maintained at 1.5 and 2.6 ml, respectively, throughout the transport experiment by replacing the volume (100 µl) removed from the receiver chamber at each sampling point with an equal volume of blank transport buffer. In addition, a 100 µl sample was taken from the donor chamber at the beginning of the experiment, to confirm the initial donor concentration (C_0), and at the end of the experiment, to facilitate mass balance calculations. For apical-to-basolateral (A→B) transport, the apical chamber is the donor chamber and the basolateral chamber is the receiver chamber. Conversely, for basolateral-to-apical (B→A) transport, the basolateral chamber is the donor chamber and the apical chamber is the receiver chamber. For P-gp inhibition studies, the MDCKII-MDR1 cells were pre-incubated with the P-gp inhibitor on both sides of the monolayer for 30 min before addition of the antidepressant, and transport experiments were carried out in the presence of the P-gp inhibitor in both chambers throughout the experiment. Fresh transport buffer containing the appropriate P-gp inhibitor was replaced in the receiver side after sampling.

3.2.6. Transport experiment calculations

The apparent permeability coefficient (P_{app}; unit: $cm.s^{-1}$) of antidepressant in each individual well was calculated using equation (3.1) (Youdim *et al.*, 2003). Importantly, this equation accounts for cellular retention of the test compound, which may influence the results obtained from *in vitro* transport studies and therefore needs to be taken into consideration.

$$P_{app} = \frac{V_D}{[A \times (M_D - M_{cells})]} \times \frac{\Delta M_R}{\Delta t} \quad (3.1)$$

where, V_D = donor chamber volume (cm^3), A = surface area of the cell monolayer (4.67 cm^2), M_D = initial amount of drug in the donor chamber (mol), M_{cells} = amount

of drug retained by the membrane/cells (mol) and $\frac{\Delta M_R}{\Delta t}$ = change in the amount of drug in the receiver chamber over time (mol.s^{-1}).

The transport ratio (TR), which compares drug permeability in the B→A direction to drug permeability in the A→B direction, was calculated using equation (3.2). A TR greater than 1 indicates that net efflux of the drug is occurring. A TR value of ≥ 2 indicates that the test compound is a transported P-gp substrate, while a TR between 1.5 and 2 is inconclusive (Polli *et al.*, 2001; Zhang *et al.*, 2006).

$$TR = \frac{P_{app,B\rightarrow A}}{P_{app,A\rightarrow B}} \quad (3.2)$$

The corrected transport ratio (cTR), calculated using equation (3.3), takes the effects of endogenous transporters on drug transport across MDCK cells into account. Hence, in theory, the effect of human P-gp is evaluated specifically. However, transfection with *ABCB1* may alter the expression of endogenous transporters (Kuteykin-Teplyakov *et al.*, 2010). Therefore, a cTR value of ≥ 1.5, in conjunction with a reduction in TR in MDCKII-MDR1 cells on co-incubation of a P-gp inhibitor, is required for confirmation that the test compound is a transported P-gp substrate (Polli *et al.*, 2001; Schwab *et al.*, 2003).

$$cTR = \frac{TR_{(MDCKII-MDR1)}}{TR_{(MDCKII-WT)}} \quad (3.3)$$

3.2.7. Analytical procedures

Samples were analysed by HPLC on a system consisting of a Shimadzu LC-20AD XR Prominence Pump, CBM-20A communication bus module, SIL-20AC XR Prominence Autosampler, CTO-20A Prominence Column oven, SPD-10A UV detector and RF-10A XL fluorescence detector (all supplied by Mason Technology, Cork, Ireland) or ESA Coulochem III with 5041 Amperometric Cell electrochemical detector (supplied by ESA Analytical, Ltd., Aylesbury, Bucks, England). All samples were injected onto a reversed phase Luna 3 µm C18 (2) 150 x 2 mm column (Phenomenex), which was protected by Krudkatcher Ultra in-line filters (Phenomenex).

The HPLC methods were adapted from previously described methods (Frahnert *et al.*, 2003; Rao *et al.*, 2011; O'Brien *et al.*, 2012a). Briefly, the mobile phase which was used on the HPLC system consisted of a mixture of 25 mM potassium dihydrogen phosphate (25 mM, pH 7 with 4N NaOH) and HPLC grade acetonitrile (56:44). Mobile phase was filtered through Millipore 0.22 µm Durapore filters (Millipore, Ireland) and vacuum degassed prior to use. Samples in transport buffer were diluted 50:50 with mobile phase prior to injection. Compounds were eluted individually using an isocratic method over a 15 min runtime at a flow rate of 0.4 $ml.min^{-1}$ after 20-40 µl injections. The column was maintained at a temperature of $30^{o}C$ and samples/standards were kept at $8^{o}C$ in the cooled autoinjector prior to analysis. The detector used and detection conditions for each antidepressant are listed in Table 3.2, along with limits of detection (LOD) and limits of quantification (LOQ) for each drug.

Table 3.2: HPLC detection conditions and limits of quantification/detection for each of the five antidepressants

Drug	Method of detection	Detection conditions	Injection volume (µl)	LOD (nM)	LOQ (nM)
Amitriptyline	UV	230 nm	40	6.6	32.9
Duloxetine	UV	230 nm	40	3.2	15.9
Fluoxetine	UV	230 nm	40	4.7	23.3
Imipramine	ECD	Potential: 600 mV; Range: 50 nA	20	0.4	1.9
Mirtazapine	FLD	Excitation: 290 nm; Emission: 370 nm	20	35.4	177.7

C_0 = initial concentration in the donor chamber; ECD: electrochemical detection; FLD: fluorescent detection; LOD: limit of detection; LOQ: limit of quantification; UV: ultraviolet detection

3.3. Results

3.3.1. Imipramine is a transported substrate of human P-gp *in vitro*

Initial experiments, carried out at 5 µM donor concentration, indicated that imipramine may have been a transported substrate of human P-gp. Imipramine permeability was greater in the B→A than A→B direction in MDCKII-MDR1 cells (TR = 1.35; Table 3.3; Figure 3.2), while there was no net efflux in MDCKII-WT cells (TR = 0.90; Table 3.3; Figure 3.2). These results yielded a cTR of 1.51, which is in excess of the cut-off for identification of a potential P-gp substrate (Figure 3.2). However, the results were not conclusive as the TR in MDCKII-MDR1 cells was below 1.5.

Therefore, additional transport experiments were undertaken at a lower donor concentration (1 µM) of imipramine to further investigate its potential efflux by human P-gp. At 1µM donor concentration of imipramine, a greater net efflux effect was observed in MDCKII-MDR1 cells (TR = 1.63; Table 3.3; Figure 3.2), while there remained no difference between imipramine permeability across monolayers of MDCKII-WT cells in the A→B and B→A directions (TR = 0.97; Table 3.3; Figure 3.3), resulting in a cTR of 1.68 (Figure 3.3).

As the TR in MDCKII-MDR1 cells was above 1.5 and the cTR indicated that P-gp efflux of imipramine was taking place, transport experiments were repeated in the presence of the P-gp inhibitors verapamil (200 µM) or CsA (25 µM). Co-incubation with verapamil attenuated the directional efflux effect, reducing the TR in MDCKII-MDR1 cells to 1.11 (Table 3.3; Figure 3.3). Similarly, co-incubation with CsA attenuated the directional efflux effect, reducing the TR in MDCKII-MDR1 cells to 1.05 (Table 3.3; Figure 3.3). Taken together, these results indicate that imipramine is a transported substrate of human P-gp.

3.3.2. Human P-gp had no effect on the permeability of amitriptyline, duloxetine, fluoxetine or mirtazapine *in vitro*

3.3.2.1. Amitriptyline

The permeability of amitriptyline was equivalent in A→B and B→A directions in both MDCKII-WT (TR = 0.97) and MDCKII-MDR1 cells (TR = 0.87; Table 3.3; Figure 3.2), thus yielding a cTR of 0.90 (Figure 3.2). These results indicate that human P-gp had no impact on amitriptyline transport in this model.

3.3.2.2. Duloxetine

The permeability of duloxetine was equivalent in both directions in MDCKII-WT cells (TR = 0.93; Table 3.3; Figure 3.2). However, in MDCKII-MDR1 cells, the permeability of duloxetine was greater in the A→B direction than in the B→A direction (TR = 0.72; Table 3.3; Figure 3.2), resulting in a cTR of 0.78 (Figure 3.2). These results indicate that human P-gp had no impact on duloxetine transport in this model, but may be subject to uptake processes in MDCKII-MDR1 cells.

3.3.2.3. Fluoxetine

The permeability of fluoxetine across both MDCKII-WT (TR = 0.80) and MDCKII-MDR1 (TR = 0.63) cells was greater in the A→B direction than in the B→A direction (Table 3.3; Figure 3.2), respectively, resulting in a cTR of 0.78 (Figure 3.2). These results indicate that fluoxetine is not a transported substrate of human P-gp in this model, but may be subject to uptake processes by endogenous canine kidney transporters in both MDCKII-WT and MDCKII-MDR1 cells.

3.3.2.4. Mirtazapine

The permeability of mirtazapine was marginally greater in the B→A than A→B direction in both MDCKII-WT and MDCKII-MDR1 cells. However, the TRs were equivalently low in both cases (1.12 and 1.16, respectively; Table 3.3; Figure 3.2), yielding a cTR of 1.04 (Figure 3.2). These results indicate that mirtazapine is not a transported substrate of human P-gp in this model.

Table 3.3: Permeability values calculated for each antidepressant drug from *in vitro* bidirectional transport studies

Drug	Donor concentration	Cell type	P-gp inhibitor	P_{app} A→B ($cm.s^{-1}$ x 10^{-5})	P_{app} B→A ($cm.s^{-1}$ x 10^{-5})
Amitriptyline	5 µM	MDCKII-WT	-	2.47 ± 0.09	2.40 ± 0.04
		MDCKII-MDR1	-	2.10 ± 0.33	1.83 ± 0.18
Duloxetine	5 µM	MDCKII-WT	-	2.18 ± 0.14	2.02 ± 0.08
		MDCKII-MDR1	-	1.48 ± 0.17	1.07 ± 0.10
Fluoxetine	5 µM	MDCKII-WT	-	2.32 ± 0.16	1.87 ± 0.19
		MDCKII-MDR1	-	1.91 ± 0.12	1.20 ± 0.07
Imipramine	5 µM	MDCKII-WT	-	1.94 ± 0.22	1.74 ± 0.26
		MDCKII-MDR1	-	1.56 ± 0.11	2.11 ± 0.19
	1 µM	MDCKII-WT	-	2.04 ± 0.00	1.98 ± 0.40
		MDCKII-MDR1	-	1.40 ± 0.21	2.28 ± 0.07
		MDCKII-MDR1	Verapamil	1.48 ± 0.12	1.65 ± 0.10
		MDCKII-MDR1	CsA	1.62 ± 0.03	1.70 ± 0.14
Mirtazapine	5 µM	MDCKII-WT	-	2.05 ± 0.11	2.29 ± 0.08
		MDCKII-MDR1	-	1.96 ± 0.05	2.29 ± 0.01

P_{app}: apparent permeability (n = 3; mean ± SD); TR: transport ratio; cTR: corrected transport ratio

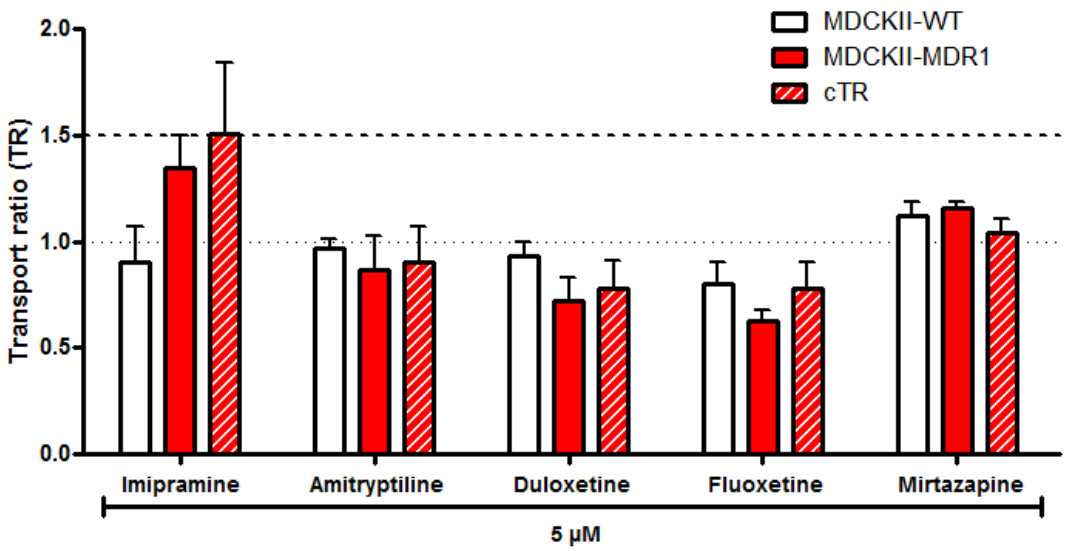


Figure 3.2: *Transport ratios (TR) in MDCKII-WT (white bars) and MDCKII-MDR1 cells (black bars), as well as corrected transport ratio (cTR) values (black bars with white diagonal stripes), for each drug at 5 µM donor concentration (mean ± SD; n = 3). Imipramine was the only drug to exhibit net efflux in MDCKII-MDR1 cells (TR = 1.35 and cTR = 1.51), indicating that it may be a transported substrate of human P-gp.*

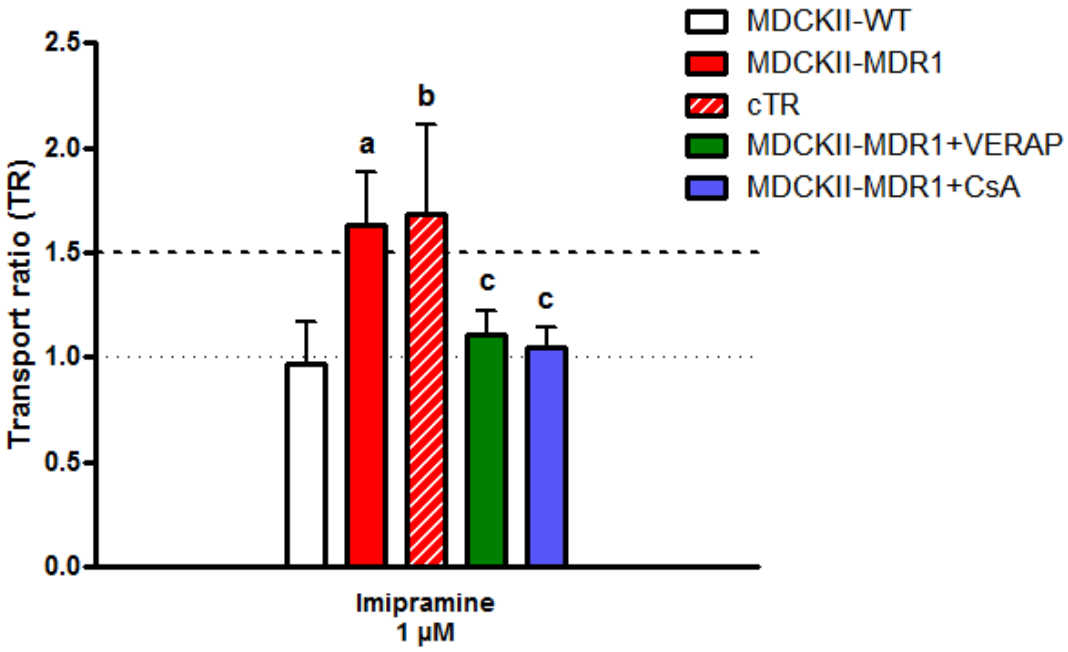


Figure 3.3: *Transport ratios (TR) in MDCKII-WT (white bar) and MDCKII-MDR1 cells (black bar), as well as the corrected transport ratio (cTR) value (black bar with white diagonal stripes), for imipramine at 1 µM donor concentration (mean ± SD; n = 3). Imipramine exhibited net efflux in MDCKII-MDR1 cells (TR = 1.63 and cTR = 1.68). Transport experiments were repeated in the presence of the P-gp inhibitors verapamil (grey bar with horizontal black lines) or CsA (grey bar with black squares) to determine if P-gp efflux was responsible for its elevated TR.*

[a] A compound with a TR of 1.5–2.0 is classified as a P-gp substrate if the directional efflux is attenuated by the presence of a P-gp inhibitor (Polli et al., 2001)

[b] Above pre-defined cTR threshold for identification of a P-gp substrate (Schwab et al., 2003)

[c] Directional efflux is attenuated by the presence of a P-gp inhibitor

3.4. Discussion

Rodent studies have recently demonstrated that brain levels of the tricyclic antidepressant (TCA) imipramine can be augmented in rats by pre-treatment with a P-gp inhibitor (Clarke *et al.*, 2009; O'Brien *et al.*, 2012a, *Chapter 2*). The present study using the MDCKII-MDR1 cell-line has revealed that imipramine is also a transported substrate of human P-gp. Taken together, these findings indicate that P-gp may restrict imipramine transport across the BBB in humans. In addition, the present findings serve to clarify conflicting evidence from previous studies as to whether imipramine is a transported substrate of human P-gp (Mahar Doan *et al.*, 2002; Faassen *et al.*, 2003).

Regarding the previous studies, a TR of 6.04 has been reported for imipramine in transport experiments in the Caco-2 cell line, indicating that active efflux processes were limiting imipramine transport in the A→B direction (Faassen *et al.*, 2003). However, no further experiments to elucidate the transporter(s) responsible for this effect were reported, which is vital as the Caco-2 cell line expresses a wide variety of transporters in addition to P-gp (Taipalensuu *et al.*, 2001). In contrast, a TR of 1.05 was previously reported for imipramine in the MDCKII-MDR1 cell-line (Mahar Doan *et al.*, 2002). The discrepancy with present findings may be related to differences in the methodological approaches between the two studies. For example, the use of an appropriate donor concentration is a key factor in obtaining accurate results using *in vitro* bidirectional transport studies. At higher donor concentrations, P-gp may become saturated, leading to functional knockout of P-gp activity (Balimane *et al.*, 2006). This may result in false negative findings, even for well-known P-gp substrates (Balimane *et al.*, 2006). To avoid this pitfall, it has been recommended that donor concentrations of 5 µM or less should be used where the sensitivity of the analytical assay permits (Balimane *et al.*, 2006). Whereas the previous work of Mahar Doan and colleagues consisted of experiments involving a 10 µM donor concentration of imipramine, two different lower concentrations of imipramine were used in the present study (5 µM and 1 µM). At 5 µM donor concentration we observed a trend towards P-gp efflux for imipramine, leading us

to carry out further experiments at 1 µM. At this lower donor concentration of imipramine (1 µM = 280 ng.ml^{-1}), which reflects therapeutic plasma levels (175-350 ng.ml^{-1}) (Perry *et al.*, 1994), a clear P-gp efflux effect was observed. In addition, the previous transport experiment was conducted over a shorter timeframe, with permeability calculations based on samples taken a single time-point at one hour (Mahar Doan *et al.*, 2002), whereas the present study involved samples taken at several time-points over two hours. It is also important to note that monolayer TEER values were not reported in the previous paper (Mahar Doan *et al.*, 2002). As a result, it is not possible to compare the confluencies of the monolayers between the earlier work and our present studies.

In contrast to imipramine (TR = 1.35 in MDCKII-MDR1 cells and cTR = 1.51 at 5 µM donor concentration), there was no indication that any of the other antidepressants included in the present study may be subjected to efflux by human P-gp when tested at a 5 µM donor concentration (TR ≤ 1.16 in MDCKII-MDR1 cells and cTR ≤ 1.04 in each case). These results suggest that none of these antidepressant drugs are meaningfully transported substrates of human P-gp. Therefore, further transport experiments using additional donor concentrations for each of these antidepressants were not carried out.

The observation that amitriptyline, another TCA which is structurally very similar to imipramine (Table 3.1), was not a transported substrate of human P-gp in our *in vitro* bidirectional transport model reveals that findings in relation to imipramine do not generalize to other antidepressants, even those from the same class with very similar molecular structures. This emphasizes the need to experimentally determine interactions between P-gp and drugs, as attempts at prediction based on structural and physicochemical properties alone can be fraught with difficulties. Historically, efforts to develop quantitative structure-activity relationships for P-gp substrates have been challenging (Sharom, 2008). However, recent developments, involving larger datasets and taking a complex range of parameters into consideration, have yielded more promising results, and may prove to be useful screening tools in the early stages of drug development (Desai *et al.*, 2013). The number of hydrogen

bond acceptors (HBAs) in a molecule has been identified as one of the key properties involved, with an increased number of HBAs associated with a higher likelihood of being a P-gp substrate (Desai *et al.*, 2013). Therefore, it is plausible that the additional HBA provided by the nitrogen atom in the middle ring of the imipramine structure, as opposed to the carbon atom in the analogous position of the amitriptyline structure (Table 3.1), may explain why imipramine was found to be a transported substrate of human P-gp in the present studies, but amitriptyline was not.

The existing rodent data in relation to the influence of P-gp on amitriptyline brain distribution is inconclusive. While it seems that P-gp does limit brain levels of amitriptyline in mice (Uhr *et al.*, 2000), this effect appears to be transient and dependent on the dosing regimen used (Grauer and Uhr, 2004; Uhr *et al.*, 2007). Furthermore, previous *in vitro* transport investigations pertaining to the efflux of amitriptyline by human P-gp have yielded antithetical findings. In common with imipramine, it has been reported that amitriptyline may be a transported substrate of human P-gp in Caco-2 cells (TR = 4.51) (Faassen *et al.*, 2003), while others concluded that it was not following transport experiments in MDCKII-MDR1 cells (TR = 1.34) (Mahar Doan *et al.*, 2002). Therefore, the present finding that amitriptyline was not a transported substrate of human P-gp helps to bring clarity to the role of P-gp in amitriptyline distribution.

In common with several other antidepressant drugs, most notably paroxetine and sertraline (Weiss *et al.*, 2003), the serotonin and noradrenaline reuptake inhibitor (SNRI) duloxetine has been reported to be an inhibitor of P-gp (Ruike *et al.*, 2010). However, to our knowledge, there has been no study to date, either *in vivo* or *in vitro*, which has investigated if duloxetine is subject to P-gp efflux. Therefore, the finding that human P-gp had no effect on duloxetine permeability in our *in vitro* model offers novel insight into the interaction between duloxetine and P-gp. This finding is particularly important given duloxetine's increasing clinical importance and its role in a wide range of indications in addition to depression. Interestingly, *in vivo* studies found that P-gp limits brain levels of another SNRI, venlafaxine (Doran

et al., 2005; Karlsson *et al.*, 2010). However, in common with duloxetine, venlafaxine was not a transported substrate of human P-gp *in vitro* (Feng *et al.*, 2008). This highlights potential species differences and again underlines the importance of analysis involving human P-gp such as that reported in the present study.

There are directly conflicting reports in the literature pertaining to the role of P-gp in the transport of the selective serotonin reuptake inhibitor (SSRI) fluoxetine across the BBB in mice (Uhr *et al.*, 2000; Doran *et al.*, 2005). This discrepancy may be attributable to differences between single P-gp knockout strains [e.g. *abcb1a*$^{(-/-)}$] and double P-gp knockout strains [i.e. *abcb1ab*$^{(-/-)}$), as recently highlighted in relation to the brain accumulation of glucocorticoids (Mason *et al.*, 2012). However, two *in vitro* transport studies have concurred, reporting that fluoxetine was not a transported substrate of human P-gp in MDCKII-MDR1 cells (Mahar Doan *et al.*, 2002; Feng *et al.*, 2008). The present study corroborates these earlier *in vitro* transport studies, indicating that P-gp is unlikely to influence the brain distribution of fluoxetine in patients.

The present finding that mirtazapine was not a transported substrate of human P-gp in our *in vitro* model correlates well with *in vivo* studies, in which there was no difference in brain levels of mirtazapine between wild-type and P-gp knockout mice (Uhr *et al.*, 2003; Uhr *et al.*, 2008). While several studies have highlighted potential pharmacogenetic interactions between single nucleotide polymorphisms (SNPs) in *ABCB1* and response to antidepressant treatment (Gex-Fabry *et al.*, 2008; Kato *et al.*, 2008; Nikisch *et al.*, 2008; Lin *et al.*, 2011; Singh *et al.*, 2012), there has been little consistency regarding the involvement of particular SNPs. Moreover, attempts to replicate some of these positive studies have failed (Mihaljevic Peles *et al.*, 2008; Peters *et al.*, 2008). In addition, no similar pharmacogenetic association has been found for other antidepressants (Roberts *et al.*, 2002; Laika *et al.*, 2006; Perlis *et al.*, 2010). The often contradictory literature surrounding the role of genetic variations of *ABCB1* in antidepressant response is complicated by several factors, including the lack of clarity regarding the P-gp substrate status of the antidepressants involved in

many of the studies. In two recent investigations, the antidepressants involved were categorised as P-gp substrates or non-substrates (Uhr *et al.*, 2008; Sarginson *et al.*, 2010). These studies reported significant associations between one particular SNP (rs2032583) and response to treatment with antidepressants that were P-gp substrates, while there was no such association for non-substrates of P-gp (Uhr *et al.*, 2008; Sarginson *et al.*, 2010). Based on findings from experiments involving P-gp knockout mice (Uhr *et al.*, 2003; Uhr *et al.*, 2008), mirtazapine was used as the non-P-gp-substrate control in these studies. However, to our knowledge, there were no data pertaining to human P-gp available for mirtazapine prior to the present study. In light of well-documented species differences in P-gp activity and substrate specificity (Yamazaki *et al.*, 2001; Katoh *et al.*, 2006; Baltes *et al.*, 2007; Syvanen *et al.*, 2009), it was vital that further experiments to confirm that mirtazapine is not a transported substrate of human P-gp were conducted to facilitate informed interpretation of these clinical studies. In addition, the finding that amitriptyline, duloxetine and fluoxetine were not transported substrates of human P-gp in our *in vitro* bidirectional transport model may help to explain why no association was found between *ABCB1* SNPs and response to these antidepressants in previous clinical studies (Roberts *et al.*, 2002; Laika *et al.*, 2006; Perlis *et al.*, 2010). In contrast, two studies have reported pharmacogenetic associations between *ABCB1* SNPs and response to the SSRI escitalopram (Lin *et al.*, 2011; Singh *et al.*, 2012), and we have recently identified escitalopram as a transported substrate of human P-gp in the same *in vitro* model as used in the present study (O'Brien *et al.*, 2013b, *Chapter 4*). Moreover, we also demonstrated that inhibition of P-gp enhances the brain distribution and antidepressant-like activity of escitalopram in rodents (O'Brien *et al.*, 2013b, *Chapter 4*). Taken together, these data indicate that the P-gp inhibition strategy may represent a novel approach to augment escitalopram treatment clinically. In light of the present results, it is tempting to speculate that the same might apply to imipramine. However, further preclinical and clinical studies are required to evaluate the safety and efficacy of this potential strategy.

In vitro bidirectional transport studies represent the gold standard approach for the identification of transported substrates of human P-gp (Balimane *et al.*, 2006).

Nonetheless, this approach has its limitations, and it is necessary to be cognisant of these when interpreting results from such studies. In particular, the present studies involve canine kidney MDCKII cells transfected with human P-gp. The morphological properties of this epithelial cell line differ from those of the endothelial cell-type found at the BBB (Hellinger *et al.*, 2012). Therefore, the present approach does not represent a model of P-gp interactions at the BBB *per se*. Despite this limitation, bidirectional transport experiments using MDCKII-MDR1 cells have been reported to be more effective than an *in vitro* BBB model for the identification of P-gp substrates (Hellinger *et al.*, 2012). In addition, *in vitro* bidirectional transport studies are often subject to inter-lab variability, as differences in methodological approach may influence the results obtained (Balimane *et al.*, 2006). Therefore, independent replication of findings is crucial before definitive conclusions can be safely drawn regarding the transport of drugs by human P-gp, in particular where *in vitro* results contradict *in vivo* findings. In this context, the present study offers value as it corroborates previous *in vitro* reports in relation to fluoxetine and helps to bring clarity to conflicting evidence from previous *in vitro* studies involving imipramine and amitriptyline. To further confirm the present findings, additional studies may now be warranted. In particular, studies utilising the concentration equilibrium transport assay approach, recently described in relation to anti-epileptic drugs (Luna-Tortos *et al.*, 2008; Zhang *et al.*, 2010), would be of interest.

In conclusion, the present *in vitro* bidirectional transport studies offer valuable insights into the role of P-gp in the distribution of antidepressants in humans. The results indicate that findings from previous *in vivo* studies demonstrating that the brain distribution of imipramine can be augmented by co-administration of a P-gp inhibitor in rats (Clarke *et al.*, 2009; O'Brien *et al.*, 2012a, *Chapter 2*) may translate to humans. Moreover, the present findings emphasize that results relating to imipramine and P-gp cannot be readily generalized to other antidepressants, even ones from the same class with similar structure. In addition, the present study highlights that results from studies using P-gp knockout mice may not translate to studies involving human P-gp. It is therefore important to screen individual compounds, using appropriate experimental approaches, in order to evaluate

interactions with human P-gp. Finally, results from the present studies may help to facilitate informed interpretation of previous clinical studies which have investigated links between SNPs in *ABCB1* and therapeutic response to different antidepressants.

Chapter 4:
P-glycoprotein Inhibition Increases the Brain Distribution and Antidepressant-like Activity of Escitalopram in Rodents

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Abstract

Despite the clinical prevalence of the antidepressant escitalopram, over 30% of escitalopram-treated patients fail to respond to treatment. Recent gene association studies have highlighted a potential link between the drug efflux transporter P-glycoprotein (P-gp) and response to escitalopram. The present studies investigated pharmacokinetic and pharmacodynamic interactions between P-gp and escitalopram. *In vitro* bidirectional transport studies revealed that escitalopram is a transported substrate of human P-gp. Microdialysis-based pharmacokinetic studies demonstrated that administration of the P-gp inhibitor cyclosporin A (CsA) resulted in increased brain levels of escitalopram without altering plasma escitalopram levels in the rat, thereby showing that P-gp restricts escitalopram transport across the blood-brain barrier (BBB) *in vivo*. The tail suspension test (TST) was carried out to elucidate the pharmacodynamic impact of P-gp inhibition on escitalopram effect in a mouse model of antidepressant activity. Pre-treatment with the P-gp inhibitor verapamil enhanced the response to escitalopram in the TST. Taken together, these data indicate that P-gp may restrict the BBB transport of escitalopram in humans, potentially resulting in sub-therapeutic brain concentrations in certain patients. Moreover, by verifying that increasing escitalopram delivery to the brain by P-gp inhibition results in enhanced antidepressant-like activity, we suggest that adjunctive treatment with a P-gp inhibitor may represent a beneficial approach to augment escitalopram therapy in depression.

4.1. Introduction

Escitalopram, a selective serotonin reuptake inhibitor (SSRI), is among the most commonly prescribed and clinically important antidepressants. However, over 30% of escitalopram-treated depressed patients fail to respond to treatment, with a full remission achieved in only 50% of cases (Kennedy *et al.*, 2006). Recent clinical studies have found associations between single nucleotide polymorphisms (SNPs) in *ABCB1*, the gene encoding the multidrug efflux transporter P-glycoprotein (P-gp) in humans, and escitalopram response (Lin *et al.*, 2011; Singh *et al.*, 2012). Given that drug efflux by P-gp at the blood-brain barrier (BBB) can prevent therapeutic concentrations of centrally-acting drugs from being achieved in the brain (Loscher and Potschka, 2005b), this genetic association may indicate that escitalopram efflux by P-gp at the BBB contributes to the high prevalence of treatment failure (O'Brien *et al.*, 2012b).

Therefore, findings from these clinical studies raise the intriguing possibility that augmentation of failed escitalopram treatment with adjunctive P-gp inhibition therapy may represent a novel approach to overcome treatment-resistant depression in certain patients. Furthermore, in light of recent regulatory guidance, which has recommended the limitation of escitalopram doses used clinically due to the risk of QT prolongation, especially in the elderly (Medicines and Healthcare products Regulatory Agency, 2011), it would be advantageous if it were possible to selectively enhance escitalopram delivery to the brain by P-gp inhibition. This could facilitate escitalopram dose reduction while maintaining therapeutic response, thereby helping to minimise problems associated with peripheral side-effects.

However, several questions remain to be answered before this potential therapeutic strategy becomes realised. For example, it is unknown if escitalopram is a transported substrate of human P-gp or if P-gp efflux restricts the transport of escitalopram across the BBB. Moreover, it is unknown if any putative enhancement of escitalopram brain levels by P-gp inhibition would result in an augmentation of its pharmacodynamic activity. While several studies have demonstrated that P-gp restricts the brain distribution of various antidepressants *in vivo* (Uhr et al., 2000;

Uhr and Grauer, 2003; Uhr et al., 2003; Grauer and Uhr, 2004; Ejsing and Linnet, 2005; Uhr et al., 2007; Uhr et al., 2008; Clarke et al., 2009; Karlsson et al., 2010; Bundgaard et al., 2012; O'Brien et al., 2012a), it has not yet been determined if increasing brain levels of any antidepressant due to P-gp knockout or P-gp inhibition results in an augmentation of pharmacodynamic effect in an appropriate animal model of antidepressant activity.

The present study was designed to address these outstanding questions, using a combination of *in vitro* and *in vivo* approaches to investigate pharmacokinetic and pharmacodynamic interactions between P-gp and escitalopram.

In vitro bidirectional transport studies, using cell-lines which express human P-gp (Figure 4.1A), represent the gold standard for the identification of transported substrates of human P-gp (Balimane *et al.*, 2006; O'Brien *et al.*, 2012b). This is particularly important considering well-established species differences in P-gp specificity (Yamazaki *et al.*, 2001; Katoh *et al.*, 2006; Baltes *et al.*, 2007; Syvanen *et al.*, 2009). The integrated microdialysis approach adopted in the present pharmacokinetic studies facilitated the simultaneous and repeated monitoring of plasma and brain levels of escitalopram before and after administration of a P-gp inhibitor under steady-state conditions. This approach gives a unique insight into the effect of P-gp inhibition on escitalopram transport across the BBB. Pharmacodynamic studies were carried out to determine if increasing the delivery of escitalopram to the brain by inhibition of P-gp would result in enhanced antidepressant-like activity. To this end, the tail suspension test (TST), which is among the most commonly used and well-validated preclinical models for the assessment of antidepressant activity (Cryan *et al.*, 2005a), was conducted.

4.2. Materials and methods

4.2.1. Drugs and chemicals

Acetonitrile, potassium dihydrogen phosphate and orthophosphoric acid were obtained from Fisher Scientific (Ireland). Heparin sodium solution (Wockhardt UK Ltd, UK) and cyclosporin A (CsA; Sandimmun) were purchased from Uniphar Group (Ireland). Escitalopram oxalate was purchased from Discovery Fine Chemicals (Dorset, UK). Verapamil hydrochloride and imipramine hydrochloride were obtained from Sigma-Aldrich (Ireland), as were all other chemicals, reagents and materials unless otherwise stated.

4.2.2. In vitro bidirectional transport studies

Transport experiments were conducted in MDCKII-WT and MDCKII-MDR1 cells, obtained from the Laboratory of Prof. Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands), as described in detail in the supplementary information (Section 4.5.1). The MDCKII-MDR1 cell line is transfected with *ABCB1* and expresses human P-gp on the apical membrane in a polarised fashion when cultured on an appropriate transwell support (Pastan *et al.*, 1988). Therefore transported substrates of human P-gp will have lower permeability in the apical-to-basolateral (A→B) direction than in the basolateral-to-apical direction (B→A) due to the effect of P-gp efflux at the apical membrane, exhibiting a (B→A)/(A→B) transport ratio (TR) ≥ 2 (Polli *et al.*, 2001; Zhang *et al.*, 2006).

As the MDCKII-MDR1 cell line also expresses endogenous canine kidney transporters, the TR in MDCKII-MDR1 cells can be compared to the TR in wild-type MDCKII-WT cells to yield a corrected transport ratio (cTR), which in theory isolates the effect of human P-gp. A cTR value of ≥ 1.5 indicates the test compound is a transported substrate of human P-gp (Schwab *et al.*, 2003). However, transfection with *ABCB1* may alter the expression of endogenous transporters (Kuteykin-Teplyakov *et al.*, 2010). Therefore it is important to repeat transport experiments with inhibition of P-gp to confirm that P-gp is responsible for net efflux in MDCKII-MDR1 cells. The P-gp inhibitors verapamil (200 µM) and CsA (25 µM) were used in the present study as previously described (Tang *et al.*, 2002; Taub *et al.*, 2005).

Expression of P-gp was confirmed by western blot as described in the supplementary information (Section 4.5.1.3).

4.2.3. *In vivo* pharmacokinetic studies

4.2.3.1. Animals

Male Sprague Dawley rats (Harlan Laboratories, UK), weighing 250-320 g, were used in this study (total n = 10). Animals were group-housed 4-6 animals per cage and maintained on a 12 hour light/dark cycle (lights on at 08:00 h) with food and water *ad libitum*. Room temperature was controlled at 22 ± 1°C. All procedures were carried out in accordance with EU directive 89/609/EEC and approved by the Animal Experimentation & Ethics Committee of University College Cork.

4.2.3.2. Microdialysis probe construction and calibration

Microdialysis probes were constructed as previously described (O'Brien *et al.*, 2012a, *Chapter 2*). *In vitro* probe recovery values ranged from 5.64 to 8.28% for escitalopram. Dialysate values were not corrected to account for *in vitro* recovery, as the diffusion properties of compounds in brain tissue are likely different from *in vitro* conditions. Rather, dialysate values are expressed as a percentage of steady state concentration, calculated independently for each individual animal.

4.2.3.3. Surgical procedures

To facilitate intravenous and intra-arterial drug administration, as well as the collection of serial blood samples, the jugular vein and carotid artery of each rat were catheterised using standard surgical techniques, and microdialysis probes were inserted into the prefrontal cortex (PFC: 2.7 mm anterior and 0.7 mm lateral to bregma, lowered 5 mm from dura (Paxinos and Watson, 1998), as described previously (O'Brien *et al.*, 2012a, *Chapter 2*). Post-surgery, rats were single-housed in cylindrical plexiglass containers with the probe inlet connected to a fluid swivel (Instech Laboratories, Plymouth Meeting, PA). Artificial cerebrospinal fluid (aCSF: 147 mM NaCl, 1.7 mM $CaCl_2$, 0.9 mM $MgCl_2$, and 4mM KCl) was perfused through each microdialysis probe at a rate of 1.5 µl.min^{-1}. Rats were allowed to recover overnight prior to sampling on the following day during the optimal post-surgical period (de Lange *et al.*, 2000).

4.2.3.4. *Experimental design*

Rats were separated into two groups: Escitalopram plus vehicle (Escit+VEH) and escitalopram plus CsA (Escit+CsA; n = 5 per group). To achieve and maintain steady state levels of escitalopram (Bundgaard *et al.*, 2007), a bolus dose of escitalopram (6 $mg.kg^{-1}$ i.v.) was administered to all rats at time (t) = 0 min, followed by a continuous escitalopram infusion (4 $mg.kg^{-1}.h^{-1}$ i.v.). Rats in the Escit+CsA group were treated with the P-gp inhibitor CsA (25 $mg.kg^{-1}$ intra-arterial) (O'Brien *et al.*, 2012a, *Chapter 2*) after steady state escitalopram levels had been achieved at t = 120 min. Rats in the Escit+VEH group received the same volume (2 $ml.kg^{-1}$) of vehicle (1/12 ethanol: 2/12 Cremophor EL: 9/12 saline) at t = 120 min. Microdialysis samples were collected at 20 min intervals, and stored at -80^{o}C until analysed by HPLC. Plasma samples (~ 250 µl) were collected at seven time-points: one before escitalopram administration (blank) and one at 10, 40, 80, 120, 160 and 220 min post-escitalopram bolus administration, and stored at -80^{o}C until extraction for HPLC analysis. At the conclusion of the experiment (t = 220 min), rats were euthanized by intra-arterial administration of sodium pentobarbital. Brains were subsequently removed and dissected. The right hemisphere was taken for histological verification of probe placement. The PFC and hippocampus were taken from the contralateral hemisphere to determine escitalopram concentration in brain tissue at the termination of the experiment. Escitalopram was extracted from plasma and brain tissue using a liquid-liquid extraction technique described in supplementary information (Section 4.5.2).

4.2.4. *In vivo* pharmacodynamic studies

4.2.4.1. *Tail suspension test*

The tail suspension test (TST), one of the most widely used assays for assessing antidepressant activity in rodents (Cryan *et al.*, 2005a), was carried out using male C57BL/6JOlaHsd mice (Harlan Laboratories, UK; 6-8 weeks old; total n= 67), as described previously (O'Connor and Cryan, 2013). This allowed an assessment of the pharmacodynamic impact of pre-treatment with the P-gp inhibitor verapamil on escitalopram activity. Briefly, after a 5-7 day acclimation period, mice were pre-treated with either the P-gp inhibitor verapamil (20 $mg.kg^{-1}$ i.p.) or saline one hour

before administration of either escitalopram (0.1 mg.kg^{-1} or 1 mg.kg^{-1} i.p.) or saline. Thirty minutes after the second injection, mice were individually suspended by the tail from a horizontal bar using adhesive tape. Six minute test sessions were recorded by a video camera and subsequently scored by a trained observer blind to the treatment groups. The amount of time spent immobile by the animal was recorded. Brain tissue (PFC and hippocampus) was harvested from each animal 10 min after commencement of the TST and brain escitalopram and verapamil levels were determined using the extraction procedure described in the supplementary information (Section 4.5.2).

4.2.4.2. Locomotor activity

To investigate potential locomotor effects, which would confound analysis of behavioural data from the TST, the impact of drug treatment on locomotor activity was assessed as previously described (O'Connor and Cryan, 2013). A separate cohort of mice were pre-treated with either the P-gp inhibitor verapamil (20 mg.kg^{-1} i.p.) or saline one hour before administration of either escitalopram (0.1 mg.kg^{-1} i.p.) or saline, thus resulting in 4 groups. Thirty minutes after the second injection, mice were individually placed in activity monitoring units (27 cm X 21 cm). Activity was then monitored via beam breaks for 60 minutes.

4.2.5. HPLC analysis

Samples were analysed by HPLC using a reversed phase Luna 3 µm C18(2) 150 x 2 mm column (Phenomenex). The HPLC method was adapted from previously described methods (Frahnert *et al.*, 2003; Unceta *et al.*, 2011). Briefly, the mobile phase consisted of a mixture of 25 mM potassium dihydrogen phosphate (25 mM, pH 7 with 4N NaOH) and HPLC grade acetonitrile (56:44). Compounds were eluted isocratically over a 15 min runtime at a flow rate of 0.4 ml.min^{-1} after a 20 µl injection. The UV detector was set to 230 nm and the fluorescent detector was set to 249 nm excitation and 302 nm emission. Escitalopram, imipramine (internal standard) and verapamil were identified and quantified based on their characteristic retention times and peak heights as determined by standard injections which were run at regular intervals during sample analysis. The limit of

quantification (LOQ) for escitalopram was 6.25 ng.ml^{-1}. The coefficients of variation (% CV) for the HPLC method were 9.02%, 2.04% and 0.34% at 6.25 ng.ml^{-1} (LOQ), 20 ng.ml^{-1} (typical of microdialysis samples) and 1000 ng.ml^{-1} (typical of extracted plasma or brain samples), respectively.

4.2.6. Data analysis and statistical procedures

All statistical analyses were carried out using standard commercial software (SPSS Statistics, version 20.0.0; SPSS, Inc., Chicago, IL). Plasma and dialysate concentration versus time profiles were analyzed using one-way repeated measures ANOVA. Where significant overall group or time effects were observed, unpaired *t*-test or LSD post-hoc test were used to elucidate differences between or within the groups, respectively. Statistical analyses of differences between the two groups for all other parameters pertaining to *in vivo* pharmacokinetic studies were carried out using unpaired *t*-test. Results from the TST and locomotor studies were analysed statistically by one-way ANOVA with LSD post-hoc. The strength of the association between immobility in the TST and brain escitalopram concentrations was evaluated using the Spearman rank-order correlation analysis. The criterion for statistical significance was $p \leq 0.05$.

4.3. Results

4.3.1. *In vitro* bidirectional transport studies

4.3.1.1. Western blot analysis

Western blot analysis confirmed that expression of P-gp in *ABCB1*-transfected MDCKII-MDR1 cells was higher than endogenous P-gp expression in MDCKII-WT cells (Figure 4.1B).

4.3.1.2. Human P-gp limited escitalopram transport across MDCKII-MDR1 cells

The transport ratio (TR) compares drug permeability across the cell monolayer in the basolateral-to-apical (B→A) direction to drug permeability in the apical-to-basolateral direction (A→B). Drugs which are transported substrates of human P-gp will have greater permeability in the B→A than A→B direction (i.e. TR > 1) in MDCKII-MDR1 cells, due to P-gp efflux restricting drug transport in the A→B direction only (Figure 4.1A). To account for the potential impact of endogenous transporters of MDCK cells, a corrected transport ratio (cTR) is determined relative to wild-type cells.

In MDCKII-WT cells, which are not transfected with *ABCB1* and therefore do not express human P-gp, the apparent permeability (P_{app}) of escitalopram was greater in the B→A than A→B direction, with a TR of 2.32 (Figure 4.1C and Table 4.1). This indicates that endogenous MDCK transporters exert a net efflux effect on escitalopram.

In MDCKII-MDR1 cells, which express human P-gp on the apical membrane only (Figure 4.1A), the difference between B→A and A→B P_{app} was greater than in MDCKII-WT cells, as evidenced by the TR of 7.22 and cTR of 3.11 (Figure 4.1D and Table 4.1). Therefore, both the TR and cTR for escitalopram substantially exceeded the recommended TR and cTR thresholds of 2 and 1.5, respectively, for the identification of transported substrates of human P-gp (Polli *et al.*, 2001; Schwab *et al.*, 2003; Zhang *et al.*, 2006). Moreover, co-incubation of the P-gp inhibitor verapamil (200 µM) reduced the TR in MDCKII-MDR1 cells to 1.51 (Figure 4.1E and Table 4.1). Similarly, co-incubation of another P-gp inhibitor, CsA (25 µM), reduced

the TR to 3.33 in MDCKII-MDR1 cells (Figure 4.1F and Table 4.1). Taken together, the data from this series of *in vitro* bidirectional transport experiments demonstrate that escitalopram is a transported substrate of human P-gp.

Figure 4.1: *In vitro bidirectional transport studies A Schematic illustrating the in vitro bidirectional transport assay. MDCKII-MDR1 cells express human P-gp in a polarised fashion at the apical membrane only when cultured on a transwell support. When substrates of human P-gp are added to the apical chamber, their transport across the monolayer of cells is restricted by P-gp efflux, thereby reducing their apical-to-basolateral (A→B) permeability. When substrates of human P-gp are added to the basolateral chamber, P-gp does not limit their transport across the monolayer as it is not expressed at the basolateral membrane. Therefore, human P-gp substrates will have greater permeability in the basolateral-to-apical (B→A) direction than in the A→B direction, and directional efflux is attenuated by co-incubation of a P-gp inhibitor. Bidirectional transport studies are also carried out in MDCKII-WT cells, which do not express human P-gp, to determine the influence of endogenous transporters, including canine P-gp, on drug permeability. B Representative image of western blot demonstrating over 4.5-fold greater expression of P-gp in MDCKII-MDR1 cells than MDCKII-WT cells. C-D Escitalopram accumulation in receiver chamber over time in MDCKII-WT and MDCKII-MDR1 cells. Escitalopram crossed the monolayer to a greater extent in the B→A direction than A→B direction in both MDCKII-WT (C) and MDCKII-MDR1 (D) cells, indicating net efflux in both cell lines. However, the magnitude of the efflux effect was substantially greater in MDCKII-MDR1 cells, highlighting a role for human P-gp. E-F Escitalopram accumulation in receiver chamber over time in MDCKII-MDR1 cells with co-incubation of the P-gp inhibitors verapamil (E) or CsA (F). Co-incubation with either P-gp inhibitor attenuated the directional efflux of escitalopram, thereby confirming that escitalopram is a transported substrate of human P-gp (mean ± SEM; n = 3 in all experiments).*

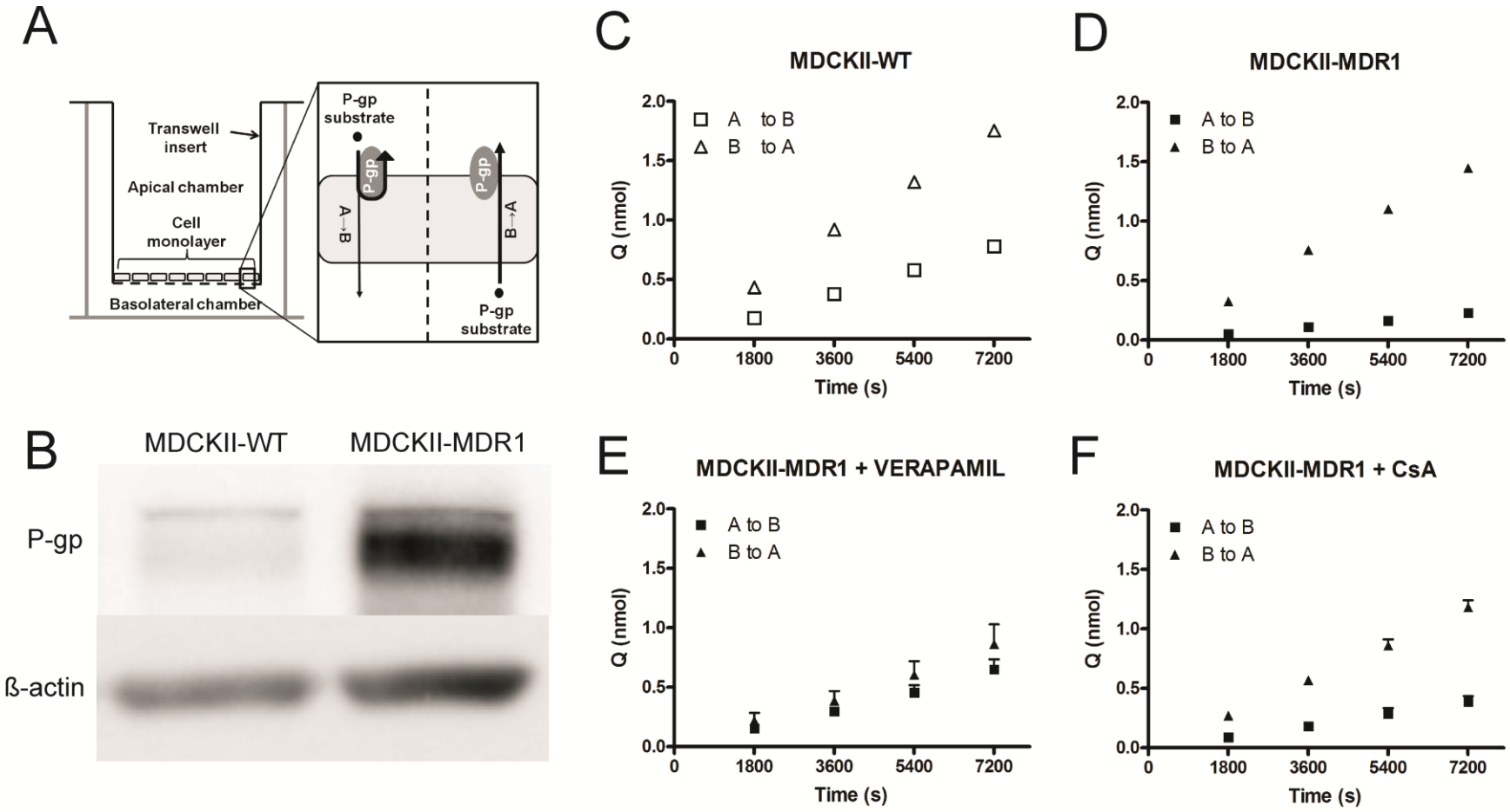

A
Transwell insert
Apical chamber
Cell monolayer
Basolateral chamber
P-gp substrate
P-gp
A→B
B→A
P-gp substrate
B
MDCKII-WT
MDCKII-MDR1
P-gp
ß-actin
C
MDCKII-WT
A to B
B to A
Q (nmol)
Time (s)
D
MDCKII-MDR1
A to B
B to A
Q (nmol)
Time (s)
E
MDCKII-MDR1 + VERAPAMIL
A to B
B to A
Q (nmol)
Time (s)
F
MDCKII-MDR1 + CsA
A to B
B to A
Q (nmol)
Time (s)

Table 4.1: Apparent permeability (P_{app}), transport ratio (TR) and corrected transport ratio (cTR) values from *in vitro* bidirectional transport studies

Cell-type	Treatment (P-gp inhibitor)	P_{app} A→B ($x10^{-6}$ $cm.s^{-1}$)	P_{app} B→A ($x10^{-6}$ $cm.s^{-1}$)	TR	cTR
MDCKII-WT	None	7.75 ± 0.33	18.00 ± 0.51	2.32	n/a
MDCKII-MDR1	None	2.11 ± 0.10	15.23 ± 0.51	7.22[1]	3.11[1]
MDCKII-MDR1	Verapamil (200 µM)	4.87 ± 0.65	7.36 ± 1.13	1.51	n/a
MDCKII-MDR1	CsA (25 µM)	3.35 ± 0.39	11.12 ± 0.42	3.33	n/a

[1] Exceeds pre-defined threshold for identification of a transported P-gp substrate (Polli *et al.*, 2001; Schwab *et al.*, 2003; Zhang *et al.*, 2006)

4.3.2. *In vivo* pharmacokinetic studies

4.3.2.1. P-gp inhibition had no effect on plasma escitalopram pharmacokinetics

Both CsA- and vehicle-treated groups exhibited similar plasma escitalopram pharmacokinetics, with no group effect on plasma escitalopram levels over time ($F(1, 8) = 0.104$, $p = 0.756$; Figure 4.2A). Steady-state plasma escitalopram levels were reached within 120 min. However, after administration of either vehicle or CsA at t = 120 min, an equivalent 15-20% increase in plasma escitalopram concentrations, compared to steady-state levels, was evident in both groups. As there was no difference between the groups in terms of plasma escitalopram pharmacokinetics, any differences in brain escitalopram concentrations observed between the groups can be attributed to altered BBB transport.

4.3.2.2. P-gp inhibition increased escitalopram concentration in microdialysis samples

Steady-state dialysate levels of escitalopram were achieved within 120 min in all cases. There was a statistically significant difference between the groups in terms of dialysate escitalopram profiles ($F(1, 8) = 11.569$, $p = 0.009$; Figure 4.2B). There was also a significant overall time effect ($F(7, 56) = 24.763$, $p < 0.001$), in addition to a significant group x time interaction ($F(1,56) = 10.160$, $p < 0.001$).

In the Escit+VEH group, administration of vehicle resulted in an increase in dialysate escitalopram levels relative to steady-state. This increase mirrored the 15-20% increase evident in plasma levels and reached a maximum 17.08% at the 200 min time-point, thereby indicating that administration of vehicle had no effect on the transport of escitalopram across the BBB. The increase from steady-state was statistically significant from 180 min onwards (Figure 4.2B).

In the Escit+CsA group, treatment with the P-gp inhibitor CsA resulted in an increase from steady state levels, reaching a maximum increase of 66.94% at t = 220 min with a statistically significant elevation relative to steady-state from t = 160 min onwards (Figure 4.2B). Moreover, the increase in the Escit+CsA group was significantly greater than that observed in the Escit+VEH group from t = 160 min

onwards (Figure 4.2B). Taken together, these *in vivo* plasma and microdialysis pharmacokinetic data demonstrate that inhibition of P-gp results in enhanced BBB transport of escitalopram.

4.3.2.3. Brain tissue escitalopram concentrations were increased following P-gp inhibition

At termination of the microdialysis experiment, PFC brain tissue escitalopram concentrations were over three times greater in CsA-treated animals than vehicle-treated controls ($t(8) = 8.602$, $p < 0.001$; Figure 4.2C). Moreover, the PFC brain tissue:plasma escitalopram concentration ratio was also elevated over three-fold in CsA-treated animals relative to controls ($t(5.617) = 5.617$, $p = 0.004$; Table 4.2), thus demonstrating that escitalopram transport across the BBB is significantly increased by P-gp inhibition.

Similarly, hippocampal brain tissue escitalopram concentrations were more than 2.5 times greater in CsA-treated animals than vehicle-treated controls ($t(8) = 11.047$, $p < 0.001$; Figure 4.2D). In addition, the hippocampal brain tissue:plasma escitalopram concentration ratio was elevated more than 2.75-fold in CsA-treated animals relative to vehicle-treated controls ($t(8) = 5.458$, $p = 0.001$; Table 4.2). These findings corroborate the microdialysis results, and demonstrate that inhibition of P-gp results in enhanced transport of escitalopram across the BBB.

Figure 4.2: *In vivo pharmacokinetic studies A Plasma escitalopram pharmacokinetics. There was no difference in escitalopram plasma pharmacokinetics between vehicle- and CsA-treated animals. Steady state plasma escitalopram levels were achieved within 120 min in both groups. Administration of vehicle or the P-gp inhibitor CsA resulted in an equivalent 15-20% increase in plasma escitalopram levels from steady state in both groups. B Dialysate escitalopram pharmacokinetics. Steady state dialysate concentrations of escitalopram were also achieved in both groups within 120 min. Administration of vehicle resulted in a significant 17% increase in dialysate escitalopram concentration from steady state levels, which mirrored increases observed in plasma. Administration of the P-gp inhibitor CsA resulted in a 67% increase from steady state, which was significantly greater than that observed in the vehicle-treated group. C-D Brain tissue escitalopram concentrations. Escitalopram concentrations in brain tissue at termination of in vivo pharmacokinetic studies were significantly greater in CsA-treated animals than vehicle-treated animals in both the prefrontal cortex (C) and the hippocampus (D) (mean ± SEM; n = 5 per group for all graphs).*
** p < 0.05; ** p < 0.01; *** p < 0.001 between the groups*
 # p < 0.05; ## p < 0.01; ### p < 0.001 compared to steady-state levels

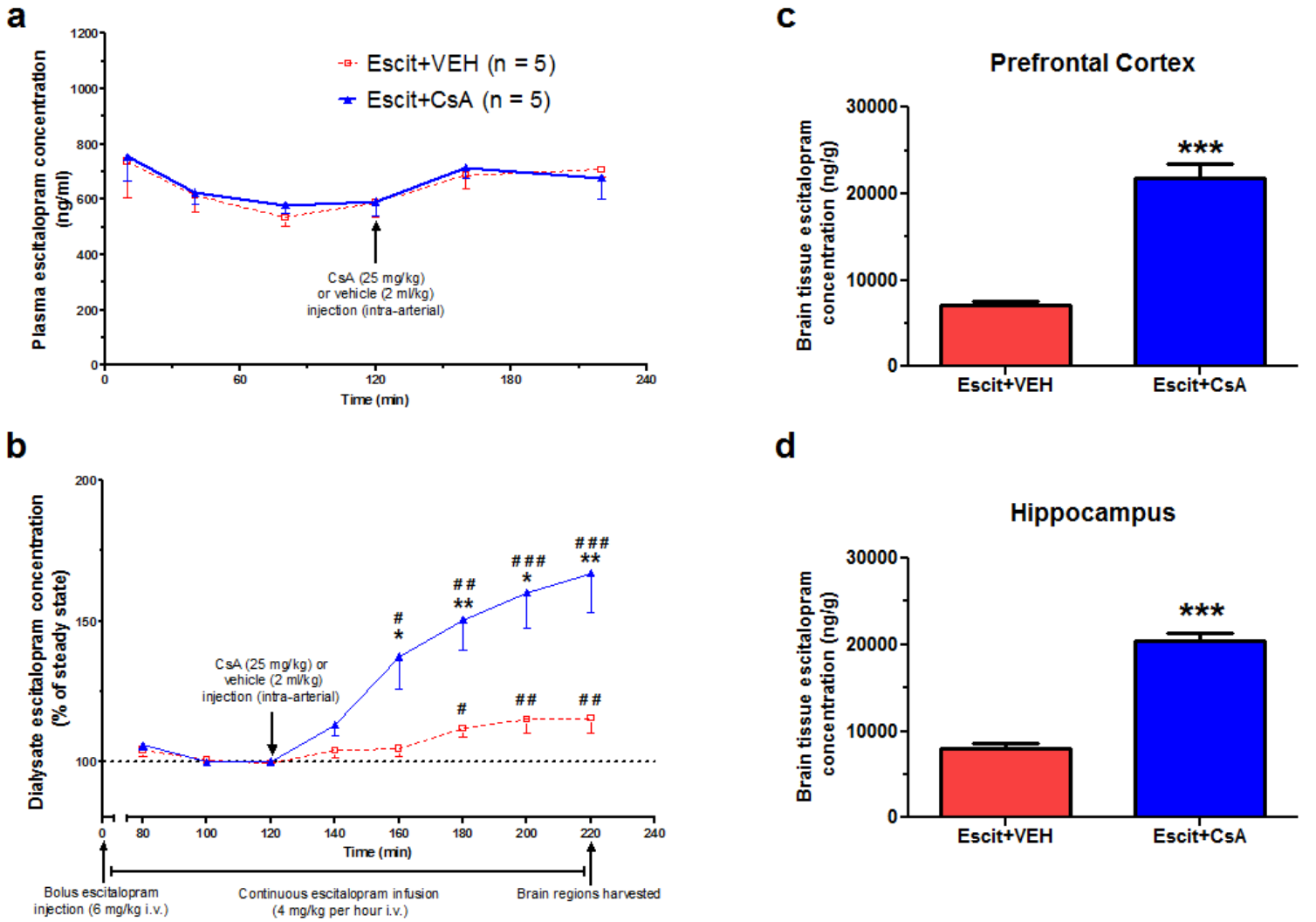

a
Plasma escitalopram concentration (ng/ml)
Escit+VEH (n = 5)
Escit+CsA (n = 5)
CsA (25 mg/kg) or vehicle (2 ml/kg) injection (intra-arterial)
Time (min)
b
Dialysate escitalopram concentration (% of steady state)
CsA (25 mg/kg) or vehicle (2 ml/kg) injection (intra-arterial)
Time (min)
Bolus escitalopram injection (6 mg/kg i.v.)
Continuous escitalopram infusion (4 mg/kg per hour i.v.)
Brain regions harvested
c
Prefrontal Cortex
Brain tissue escitalopram concentration (ng/g)
Escit+VEH
Escit+CsA
d
Hippocampus
Brain tissue escitalopram concentration (ng/g)
Escit+VEH
Escit+CsA

Table 4.2: Escitalopram concentrations in plasma and brain tissue, and brain tissue:plasma escitalopram concentration ratios at termination of *in vivo* pharmacokinetic studies

Group	Plasma	Prefrontal Cortex		Hippocampus	
	(ng.ml^{-1})	(ng.g^{-1})	B:P[1] Ratio	(ng.g^{-1})	B:P[1] Ratio
Escit+VEH	709 ± 26	7 074 ± 438	10.09 ± 0.87	7 999 ± 638	11.43 ± 1.19
Escit+CsA	678 ± 76	21 827 ± 1658	33.59 ± 4.09	20 432 ± 927	31.48 ± 3.48
p-value	0.709	< 0.001***	0.004**	< 0.001***	0.001**

[1] Brain region:plasma ratio

n = 5 per group; ** p < 0.01; *** p < 0.001

4.3.3. *In vivo* pharmacodynamic studies

4.3.3.1. Verapamil augmented escitalopram response in tail suspension test

The tail suspension test (TST) is one of the most widely used models for the assessment of antidepressant activity in mice (Cryan *et al.*, 2005a). When subjected to the short term inescapable stress of suspension by the tail, mice adopt an immobile posture. Treatment with antidepressant medications, including escitalopram (Zomkowski *et al.*, 2010), reduces the time spent immobile in this paradigm in a dose-responsive manner (Cryan *et al.*, 2005a).

In the present study, it was found that pre-treatment with the P-gp inhibitor verapamil augmented the antidepressant response at both doses of escitalopram investigated (Figure 4.3A). There was a significant overall difference between the groups in immobility in the TST ($F(5, 61) = 6.964$, $p < 0.001$; Figure 4.3A). Post-hoc analysis revealed that the 23 second reduction in immobility, compared to control animals, observed in mice treated with the lower dose of escitalopram (0.1 $mg.kg^{-1}$) without verapamil pre-treatment was not statistically significant ($p = 0.168$). Pre-treatment with verapamil augmented the response to this lower dose of escitalopram by over 60%, resulting in a statistically significant 38 second reduction in immobility *versus* controls ($p = 0.01$). Moreover, while treatment with the higher dose of escitalopram (1 $mg.kg^{-1}$) led to a significant reduction in immobility with or without pre-treatment with verapamil ($p = 0.001$ and $p < 0.001$ respectively), the magnitude of the reduction was augmented by almost 40% following pre-treatment with verapamil (Figure 4.3A). These results demonstrate that inhibition of P-gp augments the antidepressant-like effect of escitalopram in the TST.

4.3.3.2. *Effects of escitalopram and verapamil in the TST were not a function of increased locomotor activity*

Treatments which lead to increased locomotor activity could cause a reduction in immobility in the TST unrelated to antidepressant-like activity. To investigate this potential confounding factor, the effects of the present drug treatments on locomotor activity were assessed. There was a significant difference in locomotor activity between the groups ($F(3, 23) = 4.605$, $p = 0.012$; Figure 4.3B). Post-hoc analysis revealed that mice in receipt of pre-treatment with verapamil exhibited decreased locomotion relative to saline-treated controls, whether subsequently treated with saline ($p = 0.016$) or escitalopram ($p = 0.002$). Administration of escitalopram without verapamil pre-treatment had no effect on locomotor activity ($p = 0.252$). As locomotor activity was not increased by treatment with verapamil and/or escitalopram, the decreases in immobility observed in the TST can be attributed to the antidepressant-like activity of escitalopram, as opposed to being an artefact of increased locomotion.

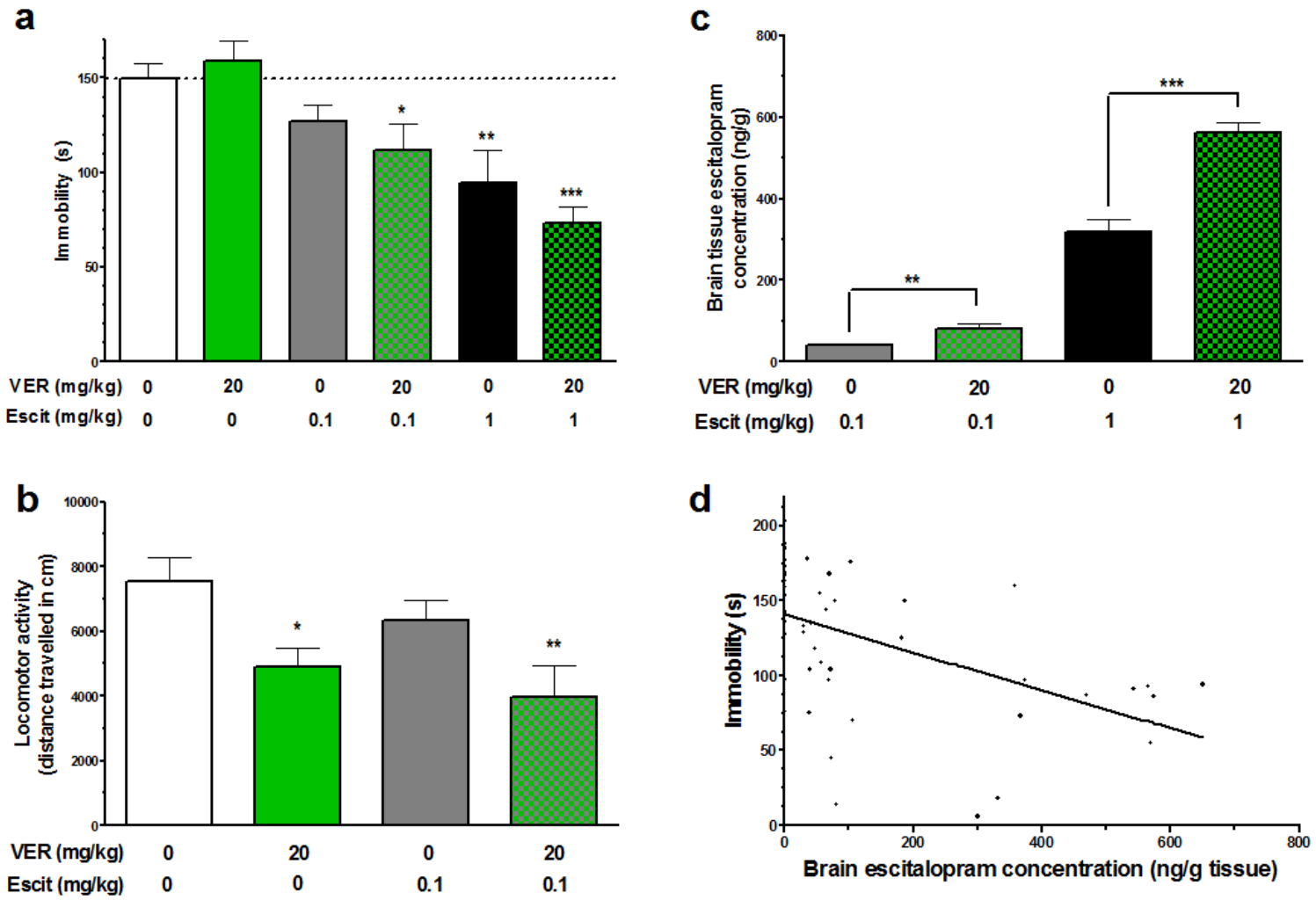
4.3.3.3. *Brain tissue concentrations of escitalopram were increased following verapamil pre-treatment*

Brain tissue escitalopram concentrations were significantly greater in mice pre-treated with the P-gp inhibitor verapamil than control mice at both doses of escitalopram treatment (Figure 4.3C). Pre-treatment with verapamil resulted in a greater than two-fold increase in brain escitalopram levels in mice treated with 0.1 $mg.kg^{-1}$ of escitalopram ($t(17) = -3.010$, $p = 0.008$) and a greater than 1.75-fold increase in brain escitalopram levels in mice treated with 1 $mg.kg^{-1}$ of escitalopram ($t(10) = -6.516$, $p < 0.001$). Verapamil concentration in brain tissue was consistent across the three verapamil-treated groups (1 163 ± 53 $ng.ml^{-1}$). These results confirm that administration of verapamil as a P-gp inhibitor resulted in increased brain levels of escitalopram in the mice during the TST.

4.3.3.4. Correlation between brain escitalopram concentration and immobility in the TST

There was a moderately strong and statistically significant negative correlation between escitalopram concentration in brain tissue and immobility in the TST (r_s = -0.594, n = 56, $p < 0.001$; Figure 4.3D). This indicates that the decreased immobility observed in escitalopram-treated mice in receipt of verapamil pre-treatment was related to the increased delivery of escitalopram to the brains of these animals.

Figure 4.3: *In vivo pharmacodynamic studies A Tail suspension test. Treatment with escitalopram reduced the time spent immobile in the TST in a dose dependent manner. Treatment with verapamil without escitalopram had no impact on immobility. However, pre-treatment with verapamil augmented the antidepressant-like effect of escitalopram in the TST, further reducing the time spent immobile at both doses of escitalopram (mean ± SEM; n = 8-17 per group). B Locomotor activity. Treatment with verapamil, escitalopram or both did not increase locomotor activity, indicating that the reductions in immobility observed in the TST were due to antidepressant-like effects. In fact, treatment with verapamil reduced locomotor activity, with or without escitalopram treatment (mean ± SEM; n = 6-7 per group). C Brain tissue escitalopram concentrations. Pre-treatment with the P-gp inhibitor verapamil before administration of escitalopram significantly increased brain concentrations of escitalopram in mice used in the TST at both doses of escitalopram treatment (mean ± SEM; n = 6-12 per group). D Correlation between brain escitalopram concentration and immobility in the TST. There was a moderately strong negative correlation between escitalopram concentration in brain tissue and immobility in the TST (rs = -0.594, n = 56, p < 0.001).*
** p < 0.05; ** p < 0.01; *** p < 0.001 relative to saline-saline control group (or between two groups where indicated in Figure 4.3C)*


a
Immobility (s)
150
100
50
0
*
**
***
VER (mg/kg) 0 20 0 20 0 20
Escit (mg/kg) 0 0 0.1 0.1 1 1
b
Locomotor activity (distance travelled in cm)
10000
8000
6000
4000
2000
0
*
**
VER (mg/kg) 0 20 0 20
Escit (mg/kg) 0 0 0.1 0.1
c
Brain tissue escitalopram concentration (ng/g)
800
600
400
200
0
**
***
VER (mg/kg) 0 20 0 20
Escit (mg/kg) 0.1 0.1 1 1
d
Immobility (s)
200
150
100
50
0
0 200 400 600 800
Brain escitalopram concentration (ng/g tissue)

4.4. Discussion

The present findings clearly demonstrate that P-gp modulates the pharmacokinetics and pharmacodynamics of the widely prescribed antidepressant escitalopram. In showing that escitalopram is a transported substrate of human P-gp, and that pharmacological inhibition of P-gp results in enhanced escitalopram transport across the BBB *in vivo,* we open up the concept that P-gp inhibition may be a viable and potentially safe strategy to augment the effects of escitalopram. Moreover, we bolster this hypothesis by showing a pharmacodynamic consequence of elevated brain levels of escitalopram by P-gp inhibition in a relevant animal model of antidepressant activity. These novel findings are especially important in light of recent clinical studies which have found associations between SNPs in *ABCB1* and response to escitalopram treatment (Lin *et al.*, 2011) and the dose of escitalopram required to achieve remission in major depression (Singh *et al.*, 2012). While there is generally no clear correlation between antidepressant plasma levels and therapeutic efficacy (Perry *et al.*, 1987; Spina *et al.*, 1997; Normann *et al.*, 2004), it has been proposed that inter-individual variability in BBB transport, due to genetic differences in P-gp functionality, may lead to clinically relevant differences in the brain distribution of certain antidepressants (Kato *et al.*, 2008).

The present results, in conjunction with previously reported pharmacogenetic associations between *ABCB1* genotype and treatment response (Lin *et al.*, 2011; Singh *et al.*, 2012), support this hypothesis in relation to escitalopram, indicating that P-gp may prevent therapeutic brain concentrations from being achieved in certain patients with elevated P-gp activity due to functional SNPs in *ABCB1*.

To our knowledge, the behavioural studies included in the present work demonstrate for the first time that the pharmacodynamic activity of an antidepressant can be augmented by inhibition of P-gp. In addition, analysis of the correlation between time spent immobile in the TST and brain escitalopram concentrations indicated that the reduction in immobility was related to the enhanced delivery of escitalopram to the brain following P-gp inhibition. These novel findings are especially exciting as they indicate that this approach to increase

escitalopram concentrations in the brain may prove to be therapeutically beneficial in patients. In previous preclinical studies, escitalopram has been shown to reduce immobility in the TST in a dose responsive manner up to a maximal response at 1 $mg.kg^{-1}$ (Zomkowski *et al.*, 2010). At higher doses of escitalopram, the time spent immobile increased, likely due to peripheral side effects, resulting in a U-shaped dose response curve (Zomkowski *et al.*, 2010). In the present study, it was found that pre-treatment with the P-gp inhibitor verapamil augmented the antidepressant response at both doses of escitalopram investigated, including an enhanced response to a 1 $mg.kg^{-1}$ dose (Figure 4.3A). This highlights that the P-gp inhibition strategy may facilitate augmentation of the central effects of escitalopram while avoiding peripheral side effects associated with higher doses of escitalopram. However, it should be noted that this approach could potentially result in increased risk of unwanted central side effects. This risk could be offset by selectively employing the P-gp inhibition augmentation strategy in patients shown to have enhanced P-gp functionality or in conjunction with low dose escitalopram, thereby serving to normalize brain levels of escitalopram rather than resulting in toxic brain concentrations.

Our finding that administration of a P-gp inhibitor increased brain concentrations of escitalopram without altering plasma levels conclusively demonstrates that inhibition of P-gp enhances escitalopram transport across the BBB. Importantly, the intracerebral microdialysis technique employed to monitor brain levels of escitalopram measures only unbound drug levels in brain extracellular fluid (de Lange *et al.*, 2000). Given that drugs are active in the unbound form and that escitalopram acts within the brain by inhibiting the serotonin reuptake transporter in the extracellular synaptic cleft, the present findings are of particular pharmacological relevance. Furthermore, the steady-state approach adopted in the present study enabled the confirmation of microdialysis findings by determining brain tissue concentrations from the same animals at termination of the experiment, when differences between the groups were maximal. To our knowledge, there are no data available in relation brain levels of escitalopram in humans in clinical practice. Therefore, it is not possible to discuss the brain

concentrations observed in the present studies in the context of those encountered clinically. Future imaging or post-mortem studies may offer further information in this regard. Nonetheless, our finding that brain tissue levels of escitalopram were increased three-fold by P-gp inhibition compares favourably with recently reported results from studies using P-gp knockout mice, which were published online as this paper was being finalised (Karlsson *et al.*, 2013). Given that it has proven difficult to reproduce effects observed in P-gp knockout mice by P-gp inhibition in the past (Ejsing and Linnet, 2005), it remained vital to demonstrate that P-gp inhibition would result in similarly increased brain levels of escitalopram before proposing that co-administration of a P-gp inhibitor may represent a promising approach to enhance escitalopram delivery to the brain. Considering that patients are treated chronically with antidepressants in clinical practice, future studies are required to investigate if escitalopram concentrations in the brain can be enhanced and maintained by repeated P-gp inhibition.

Well-documented interspecies differences in P-gp substrate specificity (Yamazaki *et al.*, 2001; Katoh *et al.*, 2006; Baltes *et al.*, 2007; Syvanen *et al.*, 2009) mean that findings in relation to P-gp transport obtained from animal models cannot be readily extrapolated to humans. Therefore, appropriate *in vitro* bidirectional transport studies involving human P-gp are necessary to enable evaluation of the clinical relevance of results from rodent studies. Indeed, this is a very pertinent point in light of our recent findings that the antidepressants amitriptyline and fluoxetine, both of which have been identified as P-gp substrates in mice (Uhr *et al.*, 2000; Doran *et al.*, 2005), are not transported substrates of human P-gp (O'Brien *et al.*, 2013a, *Chapter 3*). Therefore, the present finding that escitalopram is a transported substrate of human P-gp indicates that findings from our *in vivo* studies may translate to humans.

While the present studies have exclusively involved escitalopram, it is possible that our findings may extend to certain other antidepressant drugs, as there is a growing body of evidence to suggest that efflux by P-gp may influence treatment response to several antidepressants (reviewed in O'Brien *et al.*, 2012b). There is limited information available in relation to antidepressant transport by human P-gp *per se.*

However, in common with escitalopram, pharmacogenetic studies have revealed an association between SNPs in *ABCB1* and response to other antidepressants (Gex-Fabry *et al.*, 2008; Kato *et al.*, 2008; Nikisch *et al.*, 2008; Sarginson *et al.*, 2010). These antidepressants, including citalopram and paroxetine, are thought to be P-gp substrates based on results from studies in P-gp knockout mice (Uhr and Grauer, 2003; Uhr *et al.*, 2003; Doran *et al.*, 2005; Uhr *et al.*, 2008). However, it has been reported that similar pharmacogenetic associations do not exist for other antidepressants, including amitriptyline, duloxetine, fluoxetine and mirtazapine (Roberts *et al.*, 2002; Laika *et al.*, 2006; Uhr *et al.*, 2008; Perlis *et al.*, 2010; Sarginson *et al.*, 2010). Emerging data from our lab, indicating that these four antidepressants are not transported substrates of human P-gp, despite aforementioned preclinical findings in relation to amitriptyline and fluoxetine (Uhr *et al.*, 2000; Doran *et al.*, 2005), may offer an explanation for these observations. (O'Brien *et al.*, 2013a, *Chapter 3*). Further studies are now warranted to determine if the present findings in relation to escitalopram generalise to other antidepressants.

It should be noted that transporters other than P-gp, such as breast cancer resistance protein (BCRP) and the multidrug resistance-associated proteins (MRPs), also play an important role at the BBB. Given the significant overlap in substrate specificity between P-gp, BCRP and the MRPs (Sharom, 2008), it is possible that these alternative transporters may also influence the distribution of escitalopram across the BBB. Indeed, racemic citalopram has been reported to be an MRP1 substrate, while the same study found an association between polymorphisms in the *MRP1* gene and citalopram response (Lee *et al.*, 2010). Nonetheless, the range of approaches adopted in the present studies, not least the *in vitro* study involving MDCKII-MDR1 cells transfected with human P-gp, highlight an important role for P-gp in escitalopram pharmacokinetics and pharmacodynamics independent of any potential involvement of other transporters.

In summary, the present studies indicate that P-gp may restrict the BBB transport of escitalopram in humans, potentially resulting in sub-therapeutic brain concentrations in certain patients, particularly those with elevated P-gp

functionality due to SNPs in *ABCB1*. Moreover, the novel finding that increasing escitalopram delivery to the brain by P-gp inhibition results in enhanced antidepressant-like activity indicates that adjunctive treatment with a P-gp inhibitor may represent a beneficial approach to augment escitalopram therapy in depression in such patients. This approach could potentially have the added benefit of reducing the administered dose required to achieve escitalopram response, which is particularly desirable following recent guidance recommending the limitation of escitalopram doses used clinically due to the risk of QT prolongation (Medicines and Healthcare products Regulatory Agency, 2011). The P-gp inhibitors verapamil and CsA, used as tool compounds in the present proof-of-concept study, have the advantage of being clinically available for non-CNS indications. However, it is unlikely that these drugs would be appropriate for clinical use as P-gp inhibitors due to their lack of specificity and potency, thereby requiring high doses to adequately inhibit P-gp. Verapamil is primarily used as a calcium channel blocker, while CsA is an immunosuppressant agent which also modulates other drug transporters (Qadir *et al.*, 2005). The use of more selective second or third generation P-gp inhibitors may represent a more acceptable alternative for potential clinical use. Additional studies, both preclinical and clinical, are now warranted to further evaluate the safety and efficacy of this strategy to augment treatment response to escitalopram.

4.5. Supplementary information: supplementary materials and methods

4.5.1. *In vitro* bidirectional transport studies

4.5.1.1. *Cell-lines and materials*

MDCKII-WT and MDCKII-MDR1 cells were obtained from the Piet Borst Laboratory (Netherlands Cancer Institute, Amsterdam, The Netherlands). Transport experiments were carried out using Costar 3414 Polycarbonate Membrane Transwell® Inserts (24 mm diameter, 3 µm pore size), purchased from Sigma Aldrich, as were all other materials and chemicals unless otherwise stated.

4.5.1.2. *Cell culture*

Cells were grown in 75 cm^2 culture flasks using Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin. Cells were split twice weekly at a dilution of 1:12, and incubated at 37°C in a humidified 5% CO2 and 95% air atmosphere.

4.5.1.3. *Western blot analysis*

Expression of P-gp was confirmed by western blot analysis. Briefly, MDCKII-MDR1 and MDCKII-WT monolayers were grown as per transport experiments. Cells were washed with ice-cold phosphate-buffered saline, then lysed in lysis buffer (HEPES 20 mM; Np-40 (1%); DTT 0.5 mM; Na_3VO_4 0.1 mM; PMSF 0.1 mM; aprotinin 2 $\mu g.ml^{-1}$; leupeptin 2 $\mu g.ml^{-1}$; NaF 0.02 mM) for 15 min at 4°C. Cell lysate was centrifuged at 4°C for 10 min at 10 000 rpm. The supernatant was stored at -80°C for later analysis. Five micrograms of cell protein were loaded in 10-12%SDS-PAGE gel and run at 60-100 V for 120 min. After transfer onto a 0.2-µm nitrocellulose membrane and blocking with 5% skimmed milk and 0.1% Tween 20 in PBS, blots were probed overnight at 4°C with the C219 primary monoclonal P-gp antibody (1:100 dilution in 2% skimmed milk; Enzo Life Sciences (UK) Ltd, Exeter, UK). Reprobing was conducted for 1 h at room temperature using a goat anti-mouse IgG-HRP conjugate, diluted 1:2000 (Jackson Immunoresearch Europe Ltd). Images were obtained using a luminescent image analyzer (LAS-3000; Fujifilm, Ireland). For the detection of β-actin, the membranes were incubated with Monoclonal Anti-β-Actin–Peroxidase

antibody produced in mouse (1:15000). Immunoblots were quantified using ImageJ software.

4.5.1.4. Transport Experiments

For transport experiments, cells (passage number: 23-25) were seeded at a density of $2x10^6$ cells per well. Cells were allowed to grow to confluence over four days with 1.5 ml of medium in the apical compartment and 2.6 ml of medium in the basolateral compartment. The medium was changed daily and trans-epithelial electrical resistance (TEER) measurements were taken each day using an Endohm meter (World Precision Instruments, Sarasota, FL) to monitor the development and integrity of the cell monolayer. On the day of experiment, the mean (± SEM) TEER value for MDCKII-WT cells was 184 (± 7) $\Omega.cm^2$ and for MDCKII-MDR1 cells was 533 (± 10) $\Omega.cm^2$.Fresh cell culture medium was added to the cells two hours before the transport experiment. One hour prior to the commencement of the transport experiment, cell culture medium was removed and monolayers were rinsed twice with transport buffer (Hank's Balanced Salt Solution [HBSS] with 10 mM HEPES). Transport experiments were carried out by adding escitalopram (5 µM, freshly prepared in transport buffer) to the donor chamber and measuring the concentration of escitalopram in the receiver chamber at 30 minute intervals over a two hour incubation period at 37°C. Apical and basolateral chamber volumes were maintained at 1.5 and 2.6 ml, respectively, throughout the transport experiment by replacing the volume (100 µl) removed from the receiver chamber at each sampling point with an equal volume of blank transport buffer. In addition, a 100 µl sample was taken from the donor chamber at the beginning of the experiment, to confirm the initial concentration (C_0), and at the end of the experiment, to facilitate mass balance calculations. For apical-to-basolateral (A→B) transport, the apical chamber is the donor chamber and the basolateral chamber is the receiver chamber. Conversely, for basolateral-to-apical (B→A) transport, the basolateral chamber is the donor chamber and the apical chamber is the receiver chamber. To confirm the identification of P-gp substrates in bidirectional transport studies using MDCKII-WT and MDCKII-MDR1 cells, it is recommended that transport experiments are repeated in the presence of at least two P-gp inhibitors (Zhang *et al.*, 2006). If P-gp

transport is the mechanism net efflux in MDCKII-MDR1 cells, inhibition of P-gp will attenuate the efflux. For this reason, transport experiments were also carried out in the presence either verapamil (200 µM in transport buffer) or CsA (25 µM in transport buffer), both of which are P-gp inhibitors. The concentration chosen for each P-gp inhibitor was based on studies in the literature (Tang *et al.*, 2002; Taub *et al.*, 2005). For P-gp inhibition studies, the MDCKII-MDR1 cells were pre-incubated with the P-gp inhibitor on both sides of the monolayer for 30 min before addition of escitalopram (5 µM), and transport experiments were carried out in the presence of the P-gp inhibitor in both chambers throughout the experiment. Fresh transport buffer containing the appropriate P-gp inhibitor was replaced in the receiver side after sampling. All transport experiments were carried out in triplicate.

4.5.1.5. Transport experiment calculations

The apparent permeability coefficient (P_{app}; unit: $cm.s^{-1}$) of escitalopram in each individual well was calculated using equation (4.1) (Youdim *et al.*, 2003).

$$P_{app} = \frac{V_D}{[A \times (M_D - M_{cells})]} \times \frac{\Delta M_R}{\Delta t} \qquad (4.1)$$

where, V_D = donor volume (cm^3), A = surface area of the cell monolayer (4.67 cm^2), M_D = initial amount of escitalopram in the donor chamber (mol), M_{cells} = amount of escitalopram retained by the membrane/cells (mol) and $\frac{\Delta M_R}{\Delta t}$ = change in the amount ofe scitalopram in the receiver chamber over time ($mol.s^{-1}$).

The transport ratio (TR), which compares drug transport in the B→A direction to drug transport in the A→B direction, was calculated using equation (4.2). A ratio greater than 1 indicates that net efflux is taking place, and a TR value of ≥ 2 indicates that the test compound is a transported P-gp substrate, while a TR between 1.5 and 2 is inconclusive (Polli *et al.*, 2001; Zhang *et al.*, 2006).

$$TR = \frac{P_{app\ ,B\rightarrow A}}{P_{app\ ,A\rightarrow B}} \tag{4.2}$$

The corrected transport ratio (cTR), calculated using equation (4.3), takes the effects of endogenous transporters on drug transport across MDCK cells into account. Hence, in theory, the effect of human P-gp, is evaluated specifically. However, transfection with *ABCB1* may alter the expression of endogenous transporters (Kuteykin-Teplyakov *et al.*, 2010). Therefore, a cTR value of ≥ 1.5, in conjunction with a reduction in TR in MDCKII-MDR1 cells on co-incubation of a P-gp inhibitor, is required for confirmation that the test compound is a P-gp substrate (Polli *et al.*, 2001; Schwab *et al.*, 2003).

$$cTR = \frac{TR_{(MDCKII-MDR1)}}{TR_{(MDCKII-WT)}} \tag{4.3}$$

4.5.2. Escitalopram extraction from plasma and brain tissue

Escitalopram was extracted from plasma and brain tissue obtained from *in vivo* pharmacokinetic studies using a liquid-liquid extraction technique described previously (Clarke *et al.*, 2009), with some modifications. Briefly, 48 µl of plasma was spiked with 2 µl of the internal standard, verapamil, to yield a final concentration of 1 µg.ml^{-1} verapamil. To this verapamil-spiked plasma, 1 ml of sodium hydroxide (2 M) and 3 ml of water were added. Extraction was carried out in 7.5 ml of 1.5% isoamyl alcohol in n-heptane by vortexing for 30 seconds, followed by agitation on a mechanical shaker for 15 min and then centrifugation at 5000 rpm for 15 min at 20°C. The upper solvent layer was transferred to a tube containing 200 µl of 25 mM OPA, vortexed for 30 seconds, then agitated on a mechanical

shaker for 15 min followed by centrifugation at 5000 rpm for 15 min at room temperature. Twenty microlitres of the lower aqueous phase was injected onto the HPLC system for analysis. Brain tissue samples were weighed prior to homogenization in 500 µl of verapamil-spiked (1 $\mu g.ml^{-1}$) homogenization buffer (i.e. HPLC mobile phase). Homogenized brain tissue was centrifuged at 14 000 rpm at 8°C for 15 min, and escitalopram was extracted from 200 µl of the supernatant as described above. As verapamil was used as a P-gp inhibitor in pharmacodynamic tail suspension test (TST) studies, imipramine was used as an internal standard for extractions from samples obtained following the TST. Due to technical difficulties during this extraction procedure in brain samples from the TST, 2-4 samples per group were lost during the extraction process, meaning that drug levels could not be ascertained for these animals.

Chapter 5:

The P-glycoprotein Inhibitor Cyclosporin A Differentially Influences Behavioural and Neurochemical Responses to the Antidepressant Escitalopram

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Abstract

Recent studies have raised the possibility that P-glycoprotein (P-gp) inhibition may represent a putative augmentation strategy for treatment with certain antidepressants. Indeed, we have previously shown that administration of the P-gp inhibitor verapamil increased the brain distribution and behavioural effects of the antidepressant escitalopram. The aim of the current study was to investigate if similar effects occur with another P-gp inhibitor, cyclosporin A (CsA). CsA pre-treatment exacerbated the severity of behaviours in an escitalopram-induced mouse model of serotonin syndrome, a potentially life-threatening adverse drug reaction associated with serotonergic drugs. P-gp inhibition by CsA enhanced the brain distribution of escitalopram by 70-80%. Serotonin (5-HT) turnover in the prefrontal cortex was reduced by escitalopram, and this effect was augmented by CsA. However, CsA pre-treatment did not augment the effect of escitalopram in the tail suspension test (TST) of antidepressant-like activity. Microdialysis experiments revealed that pre-treatment with CsA failed to augment, but blunted, the increase in extracellular 5-HT in response to escitalopram administration. This blunting effect may contribute to the lack of augmentation in the TST. Taken together the present studies demonstrate that co-administration of CsA and escitalopram produces differential effects depending on the behavioural and neurochemical assays employed. Thus the results highlight the need for further studies involving more selective pharmacological tools to specifically evaluate the impact of P-gp inhibition on behavioural responses to antidepressants which are subject to efflux by P-gp.

5.1. Introduction

Currently available drug treatments for depression have unsatisfactory efficacy, with an estimated 50-60% of patients failing to adequately respond to antidepressant treatment (Fava, 2003). Hence, there is a major impetus to elucidate, and overcome, the mechanisms underlying resistance to antidepressant drug treatment. Increasing evidence from preclinical and clinical studies has highlighted a key role for the multidrug resistance efflux transporter P-glycoprotein (P-gp), expressed at the blood-brain barrier (BBB), in limiting the brain distribution, and therefore efficacy, of several antidepressant drugs (Uhr *et al.*, 2008; Clarke *et al.*, 2009; O'Brien *et al.*, 2012b; Karlsson *et al.*, 2013; O'Brien *et al.*, 2013a).

We have recently demonstrated that the selective serotonin reuptake inhibitor (SSRI) escitalopram, one of the most commonly prescribed antidepressants, is a transported substrate of human P-gp *in vitro* (O'Brien *et al.*, 2013b, *Chapter 4*). Moreover, pre-treatment with the P-gp inhibitor verapamil enhanced the brain distribution of escitalopram and augmented its antidepressant-like activity in mice (O'Brien *et al.*, 2013b, *Chapter 4*), as measured in the tail suspension test (TST), one of the most widely used models for assessing antidepressant activity in rodents (Cryan *et al.*, 2005a). In addition, microdialysis studies revealed that administration of another P-gp inhibitor, cyclosporin A (CsA), also resulted in enhanced brain levels of escitalopram in rats, thereby confirming the involvement of P-gp in its BBB transport (O'Brien *et al.*, 2013b, *Chapter 4*). These results raise the possibility that treatment with escitalopram, and potentially other antidepressants which are transported P-gp substrates, could be augmented by adjunctive therapy with a P-gp inhibitor.

Notwithstanding the potential of P-gp inhibition to enhance antidepressant brain distribution and therapeutic efficacy, the possibility that adjunctive P-gp inhibitor therapy may elicit unwanted side effects merits further consideration. Indeed, there is evidence to suggest that concurrent administration of serotonin (5-HT) enhancing antidepressants and CsA, which is a clinically used immunosuppressant as well as an inhibitor of P-gp, can result in serious adverse drug reactions. Three

case studies describing incidences of serotonin syndrome, a potentially life-threatening adverse drug reaction associated with excessive serotonergic neurotransmission (Boyer and Shannon, 2005), in patients treated with CsA and 5-HT enhancing antidepressants, including escitalopram, have been published (Wong *et al.*, 2002; Lang *et al.*, 2008; Newey *et al.*, 2011). In addition, there have been over 30 reports of CsA-related serotonin syndrome since 2001, according to the FDA Adverse Event Reporting System (http://www.ehealthme.com/ds/cyclosporine/serotonin+syndrome; Accessed 29/09/13).

In the present studies, the effect of CsA administration in an escitalopram-induced mouse model of serotonin syndrome was investigated to determine if CsA impacts on the severity of behaviours associated with this model. In addition, further behavioural studies were undertaken to determine if CsA pre-treatment would augment the antidepressant-like activity of escitalopram in the TST, as previously reported for another P-gp inhibitor (verapamil) (O'Brien *et al.*, 2013b, *Chapter 4*). Finally, the neurochemical effects of this drug combination were assessed by measuring brain tissue levels of 5-HT and its metabolite, 5-hydroxyindoleacetic acid, and by conducting intracerebral microdialysis studies to investigate the effect of pre-treatment with CsA on the increase in extracellular 5-HT in response to escitalopram administration.

5.2. Material and methods

5.2.1. Drugs and chemicals

Cyclosporin A (CsA; Sandimmun Concentrate for Solution for Infusion) was purchased from Uniphar Group (Ireland). Escitalopram oxalate was purchased from Discovery Fine Chemicals (Dorset, UK). 5-hydroxytryptophan (5-HTP) was obtained from Sigma-Aldrich (Ireland), as were all other chemicals, reagents and materials unless otherwise stated.

5.2.2. Animals

Male C57BL/6JOlaHsd mice (6-8 weeks old) and male Sprague Dawley rats (weighing 265-315 g), purchased from Harlan Laboratories, UK, were used in these studies. All animals were group-housed 4 animals per cage and maintained on a 12 hour light/dark cycle (lights on at 08:00 h) with food and water *ad libitum*. Room temperature was controlled at 22 ± 1°C. All procedures were carried out in accordance with EU directive 2010/63/EU and approved by the Animal Experimentation & Ethics Committee of University College Cork.

5.2.3. Effect of CsA administration in a mouse model of serotonin syndrome

Serotonin syndrome was evoked in male C57BL/6JOlaHsd mice by adapting previously described methods involving co-administration of the serotonin precursor 5-HTP and the SSRI escitalopram (Sanchez *et al.*, 2003; Kreilgaard *et al.*, 2008). These agents act to increase serotonergic neurotransmission by enhancing serotonin synthesis and preventing its reuptake, respectively (Haberzettl *et al.*, 2013). Together, these treatments induced a syndrome characterised by a number of specific behaviours, as outlined in Table 5.1 (Diaz and Maroteaux, 2011; Haberzettl *et al.*, 2013). Mice were pre-treated with CsA (37.5, 75 or 150 mg/kg i.p.) or its vehicle (1/12 ethanol: 2/12 Cremophor EL: 9/12 saline), one hour prior to co-administration of 5-HTP (100 mg/kg i.p.) and escitalopram (2 mg/kg i.p.) (n = 7 per group). Fifteen minutes later, mice were placed into a plexiglass arena (27 cm X 21 cm), and serotonin syndrome-related behaviours were scored by an observer blind to the treatment groups for six one-minute periods at five-minute intervals (Figure

5.1A; Table 5.1). The sum of scores for each behaviour over the course of the experiment was totalled for each animal as a measurement of the severity of each behaviour. In addition, an overall serotonin syndrome score was calculated by adding the sum of scores for each behaviour. Doses of 5-HTP and escitalopram were chosen based on pilot studies and reports that treatment with 5-HTP (100 mg/kg) induces serotonin syndrome behaviours only when administered in combination with another serotonin enhancing agent such as escitalopram (Sanchez *et al.*, 2003; Kreilgaard *et al.*, 2008).

Table 5.1: *Table listing the intermittent and continuous behaviours, and scoring systems, associated with the mouse model of serotonin syndrome used in the present study (adapted from Diaz and Maroteaux, 2011; Haberzettl et al., 2013)*

Intermittent behaviours	**Scoring**		**Continuous Behaviours**	**Scoring**	
Backward gait	0	Absent	Flat body position	0	Absent
Tics	1	Once	Piloerection	1	Perceptible
Hunched back	2	Several times	Straub tail	2	Weak
Head weaving	3	Frequently	Hind limb abduction	3	Medium
Forepaw treading	4	Permanently	Tremor	4	Maximal

5.2.4. Influence of CsA pre-treatment on the brain distribution and neurochemical and behavioural effects of escitalopram

5.2.4.1. Tail suspension test

The tail suspension test (TST), one of the most widely used models for assessing antidepressant activity in rodents (Cryan *et al.*, 2005a), was carried out using male C57BL/6JOlaHsd mice (6-8 weeks old; total n= 64 from Harlan, UK), as described previously (O'Brien *et al.*, 2013b). This facilitated investigation of the impact of pre-treatment with the P-gp inhibitor CsA on the antidepressant-like activity of escitalopram. Briefly, after a 5-7 day acclimation period, mice were pre-treated with either the P-gp inhibitor CsA (75 mg/kg i.p.) or its vehicle one hour before administration of either escitalopram (0.1 or 1 mg/kg i.p.) or saline (n = 9-14 per group). Thirty minutes after the second injection, mice were individually suspended

by the tail from a horizontal bar using adhesive tape. Six minute test sessions were recorded by video camera and the amount of time spent immobile by each animal was subsequently scored by a trained observer blind to the treatment groups. Doses of escitalopram and CsA were selected based on previous studies (Zomkowski *et al.*, 2010; O'Brien *et al.*, 2012a; O'Brien *et al.*, 2013b). Moreover, pilot studies revealed that treatment with a higher dose of CsA (150 mg/kg) resulted in a substantial increase in immobility in the TST, likely due to sedative side-effects (data not shown).

5.2.4.2. Brain concentrations of escitalopram, 5-HT and 5-HIAA

Brain tissue (prefrontal cortex (PFC) and hippocampus) was harvested from each animal 10 min after commencement of the TST. Hippocampal escitalopram levels were determined using an extraction procedure and high performance liquid chromatography (HPLC) method as described previously (O'Brien *et al.*, 2013b). Briefly, escitalopram was extracted from brain tissue using an acid-base liquid-liquid extraction technique, with imipramine as an internal standard, prior to analysis by HPLC with fluorescent detection (excitation wavelength: 249 nm; emission: 302 nm). Due to the low levels of escitalopram present in the brains of mice treated with the lower dose of escitalopram (0.1 mg/kg), it was necessary to pool hippocampal samples from two animals in these groups to successfully measure escitalopram concentrations. In addition, levels of 5-HT and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), were measured in the PFC, using previously described tissue extraction and HPLC analysis methods (Browne *et al.*, 2011; Browne *et al.*, 2013). Briefly, brain tissue was homogenized in ice cold mobile phase with N-methyl-5-HT as an internal standard prior to analysis of the supernatant by HPLC with electrochemical detection. This facilitated investigation of the effect of escitalopram and/or CsA treatment on 5-HT turnover (i.e. the 5-HIAA:5-HT ratio).

5.2.4.3. Locomotor activity

To investigate potential locomotor effects, which could confound analysis of behavioural data from the TST, the impact of drug treatment on locomotor activity was assessed as previously described (O'Brien *et al.*, 2013b). A separate cohort of mice were pre-treated with either the P-gp inhibitor CsA (75 mg/kg i.p.) or vehicle

one hour before administration of either escitalopram (0.1 mg/kg i.p.) or saline (n = 6-7 per group). Thirty minutes after the second injection, mice were individually placed in activity monitoring units (27 cm X 21 cm). Activity was then monitored via beam breaks for 60 minutes.

5.2.5. Microdialysis studies to investigate the effect of CsA pre-treatment on escitalopram-induced increases in extracellular levels of 5-HT

5.2.5.1. Microdialysis probe construction and calibration

Microdialysis probes were constructed as previously described (O'Brien *et al.*, 2012a, *Chapter 2*). *In vitro* probe recovery values ranged from ~11 – 14% for 5-HT. Dialysate values were not corrected to account for *in vitro* recovery, as the diffusion properties of compounds in brain tissue are likely different from *in vitro* conditions (Anderzhanova and Wotjak, 2013). Rather, dialysate 5-HT values are expressed as a percentage of basal concentration, calculated independently for each individual animal based on a minimum of three samples prior to drug administration, thereby facilitating comparison of relative changes in extracellular 5-HT levels following treatment (Anderzhanova and Wotjak, 2013).

5.2.5.2. Surgical procedures

Microdialysis studies were undertaken in male Sprague Dawley rats. The jugular vein of each rat was catheterised using standard surgical techniques, to facilitate intravenous drug administration, and microdialysis probes were inserted into the prefrontal cortex (PFC) (2.7 mm anterior and 0.7 mm lateral to bregma, lowered 5 mm from dura (Paxinos and Watson, 1998)), as described previously (O'Brien *et al.*, 2012a; O'Brien *et al.*, 2013b). Post-surgery, rats were single-housed in cylindrical plexiglass containers with the probe inlet connected to a fluid swivel (Instech Laboratories, Plymouth Meeting, PA). Artificial cerebrospinal fluid (147 mM NaCl, 1.7 mM $CaCl_2$, 0.9 mM $MgCl_2$, and 4mM KCl) was perfused through each microdialysis probe at a rate of 1.5 µl.min^{-1}. Rats were allowed to recover overnight prior to sampling on the following day during the optimal post-surgical period (de Lange *et al.*, 2000).

5.2.5.3. Experimental design

CsA (25 mg/kg i.v.) or vehicle were administered 30 minutes prior to escitalopram administration (0.25 mg/kg i.v. at t = 0 min) (n = 4 per group). The dose of CsA chosen was based on a previously described dosing regimen shown to result in P-gp inhibition (O'Brien *et al.*, 2012a; O'Brien *et al.*, 2013b). Microdialysis samples were collected at 20 min intervals before and after drug administration, and stored at -80^{o}C until analysed by HPLC with electrochemical detection (HPLC-ECD). At the conclusion of the experiment (t = 240 min), rats were euthanized by intravenous administration of sodium pentobarbital. Brains were subsequently removed for histological verification of probe placement.

5.2.5.4. HPLC analysis of 5-HT in microdialysis samples

The HPLC with electrochemical detection (HPLC-ECD) system consisted of a Shimadzu LC-20AD XR Prominence Pump, CBM-20A communication bus module, SIL-20AC XR Prominence Autosampler, CTO-20A Prominence Column oven (all supplied by Mason Technology, Cork, Ireland). System components were used in conjunction with Shimadzu LC solutions software (Mason Technology). The detector used was ESA Coulochem III with the 5041 Amperometric Cell (Supplied by ESA Analytical, Ltd., UK). All samples were injected onto a reversed phase Kinetex^{TM} 2.6 µm C18 100 Å (100 x 2.1 mm) column (Phenomenex, Ireland), which was protected by Krudkatcher Ultra in-line filters (Phenomenex).

The mobile phase consisted of 0.1 M citric acid, 0.1 M sodium dihydrogen phosphate, 0.01 mM EDTA (Alkem/Reagecon, Ireland), 5.6 mM octane-1-sulphonic acid and 9% (v/v) methanol (Alkem/Reagecon), adjusted to pH 2.8 with 4 N sodium hydroxide (Alkem/Reagecon), and was filtered prior to use (Millipore 0.22 µm Durapore filters, Millipore, Ireland). Compounds were eluted isocratically over a 20 minute runtime at a flow rate of 0.4 ml/min after a 20 µl injection. The samples were kept at 8˚C in the cooled autoinjector prior to injection and the column was maintained at 30˚C. The glassy carbon working electrode, combined with a platinum reference electrode (ESA Analytical, Ltd), was operated at a potential of 200 mV and a range of 100 pA. 5-HT was identified and quantified based on its

characteristic retention time and peak height, as determined by standard injections which were run at regular intervals during sample analysis.

5.2.6. Data analysis and statistical procedures

Statistical analysis of data was carried out using standard commercial software (SPSS Statistics, version 20.0.0; SPSS, Inc., Chicago, IL). The differences in brain levels of escitalopram between two groups were analysed using unpaired *t*-test. Results from 5-HT turnover, TST and serotonin syndrome studies were analysed statistically by one-way ANOVA, with LSD post-hoc where an overall group effect was determined. Differences in locomotor activity were analysed by two-way ANOVA with LSD post-hoc to elucidate statistically significant differences between treatment groups. Comparison of increases in extracellular levels of 5-HT following escitalopram administration with and without pre-treatment with CsA were analysed by repeated measures ANOVA, with parameter estimates analysis highlighting time points where significant differences existed between the groups. The criterion for statistical significance was $p \leq 0.05$.

5.3. Results

5.3.1. Effect of CsA administration in a mouse model of serotonin syndrome

5.3.1.1. Pre-treatment with CsA exacerbated the severity of behaviours associated with a mouse model of serotonin syndrome

A statistically significant difference in overall serotonin syndrome score was observed between the groups ($F(3, 24) = 24.351$, $p < 0.001$; Figure 5.1B). In addition, statistically significant differences between the groups for each of the three most prominent behaviours were observed (Figure 5.1C-E): tics ($F(3, 24) = 14.762$, $p < 0.001$), tremor ($F(3, 24) = 7.838$, $p = 0.001$) and hind limb abduction ($F(3, 24) = 14.257$, $p < 0.001$).

For the overall score, as well as for each of these individual behaviours, pre-treatment with any of the three doses of CsA used resulted in a more severe manifestation of the serotonin syndrome, as evidenced by the significantly higher scores, relative to vehicle-pre-treated controls ($p < 0.05$, 0.01 or 0.001 in each case; Figure 5.1B-E).

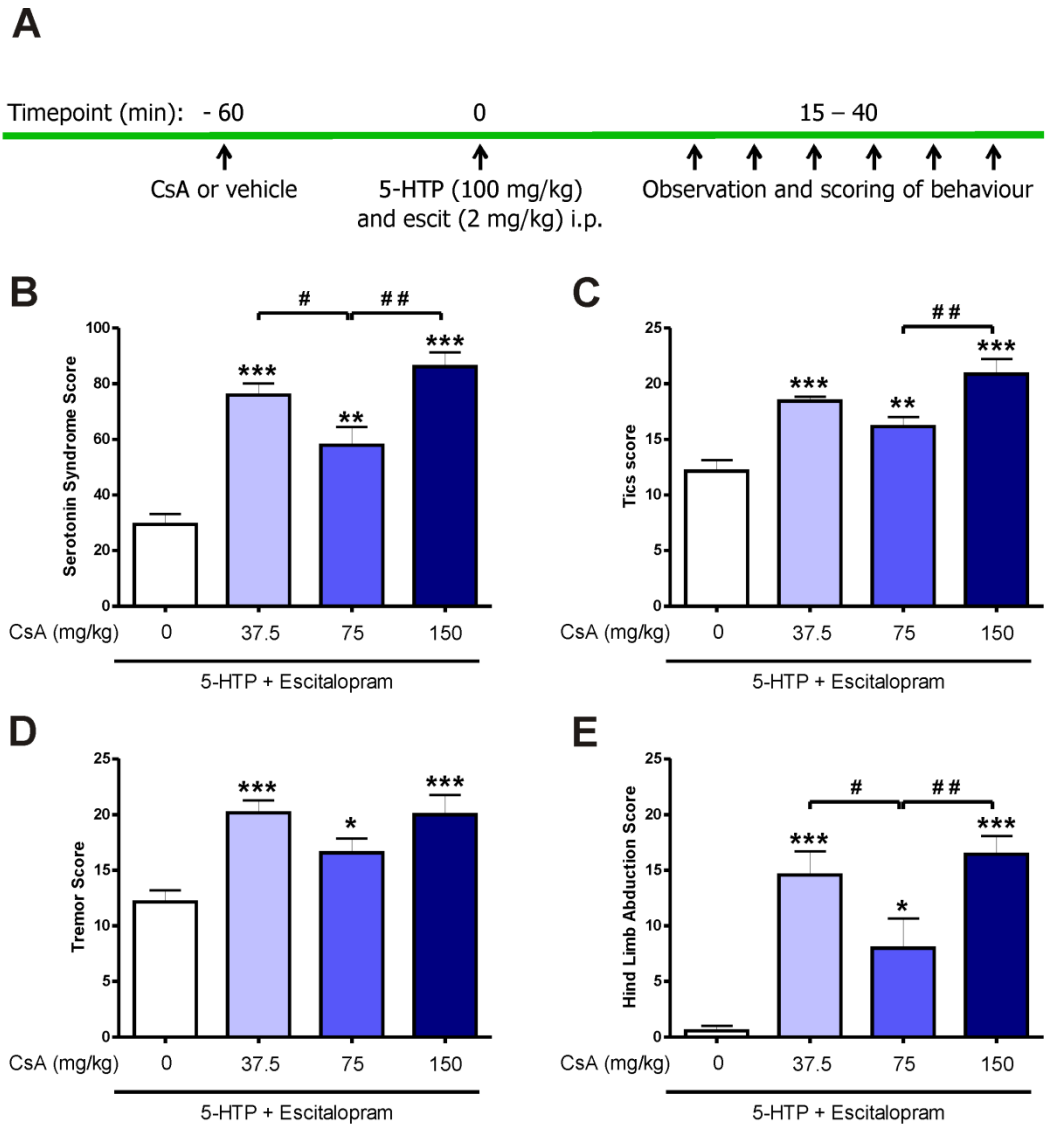

Figure 5.1: *Effect of cyclosporin A (CsA) pre-treatment in a mouse model of serotonin syndrome. A Schematic illustrating the dosing regimen and scoring protocol used. B Overall serotonin syndrome score. Pre-treatment with any of the 3 doses of CsA significantly increased the overall serotonin syndrome score, relative to vehicle-treated mice. This increase was significantly lower in mice treated with 75 mg/kg CsA compared to the other two doses of CsA. C Cumulative scores for tics behaviour. Pre-treatment with any of the 3 doses of CsA resulted in a significantly greater number of tics during the observation periods compared to vehicle-treated mice, and this effect was more substantial in mice pre-treated with 150 mg/kg than 75 mg/kg of CsA. D Cumulative scores for tremor behaviour. Pre-treatment with any of the 3 doses of CsA significantly increased the severity of tremors in the mouse model of serotonin syndrome during the observation periods, relative to the vehicle-treated group. E Cumulative hind limb abduction score. Hind limb abduction was more pronounced in mice pre-treated with CsA than vehicle. This effect was greater in mice in receipt of CsA 37.5 or 150 mg/kg than 75 mg/kg (n = 7 per group).*
** $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ relative to vehicle-pre-treated group*
$^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$ between indicated groups in receipt of different doses of CsA

5.3.2. Influence of CsA pre-treatment on the brain distribution and neurochemical and behavioural effects of escitalopram

5.3.2.1. CsA pre-treatment increased hippocampal concentrations of escitalopram

Hippocampal escitalopram concentrations were significantly greater in mice pre-treated with the P-gp inhibitor CsA than control mice at both doses of escitalopram treatment (Figure 5.2A+B). Pre-treatment with CsA resulted in a 71.4% increase in brain escitalopram levels in mice treated with 0.1 mg/kg of escitalopram ($t(4.474) = -10.147$, $p < 0.001$; Figure 5.2A) and a 79.8% increase in brain escitalopram levels in mice treated with 1 mg/kg of escitalopram ($t(16) = -7.385$, $p < 0.001$; Figure 5.2B).

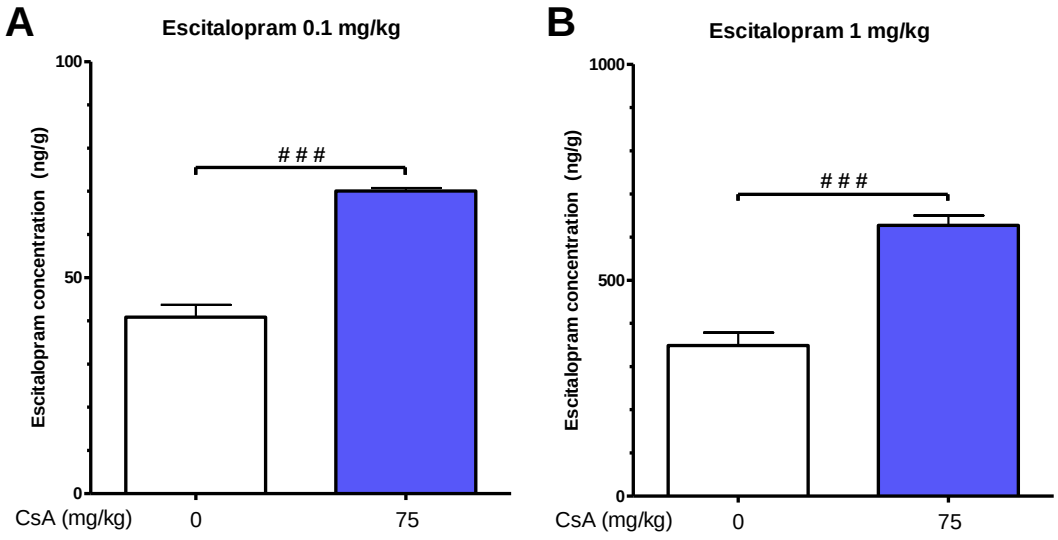

Figure 5.2: *Effect of cyclosporin A (CsA) pre-treatment on brain concentrations of escitalopram. A Pre-treatment with CsA (75 mg/kg) resulted in a 71.4% increase in hippocampal concentrations of escitalopram at a dose of 0.1mg/kg (n = 5 pooled samples per group). B Pre-treatment with CsA (75 mg/kg) resulted in a 79.8% increase in hippocampal concentrations of escitalopram at a dose of 1mg/kg (n = 9 per group).*
p < 0.001 between groups

5.3.2.2. *Pre-treatment with CsA augmented the effect of escitalopram on serotonin turnover in the PFC*

The treatment groups differed significantly in terms of 5-HT turnover in the PFC ($F(5, 60) = 13.961$, $p < 0.001$; Figure 5.3A). There was no difference in PFC 5-HT levels between the treatment groups ($F(5, 60) = 1.494$, $p = 0.205$; Figure 5.3B), but there was a significant difference in PFC 5-HIAA levels ($F(5, 60) = 2.513$, $p = 0.039$; Figure 5.3C).

In vehicle-pre-treated animals, escitalopram had no effect on either 5-HT or 5-HIAA levels in the PFC, but reduced 5-HT turnover at the higher dose of escitalopram only (1 mg/kg; $p < 0.001$).

CsA administration, without escitalopram treatment, had no impact on 5-HT levels, 5-HIAA levels or 5-HT turnover ($p > 0.05$). However, when CsA pre-treatment was followed by escitalopram administration, 5-HIAA levels were significantly reduced compared to control mice ($p = 0.019$ and 0.012 for escitalopram 0.1 and 1 mg/kg, respectively). Interestingly, at both low (0.1 mg/kg) and high (1 mg/kg) doses of escitalopram, CsA pre-treatment resulted in statistically significant reductions in 5-HT turnover relative to the control group ($p < 0.001$). Moreover, 5-HT turnover was reduced to a greater extent after CsA pre-treatment than observed following administration of the same dose of escitalopram on its own ($p < 0.01$ for both doses of escitalopram).

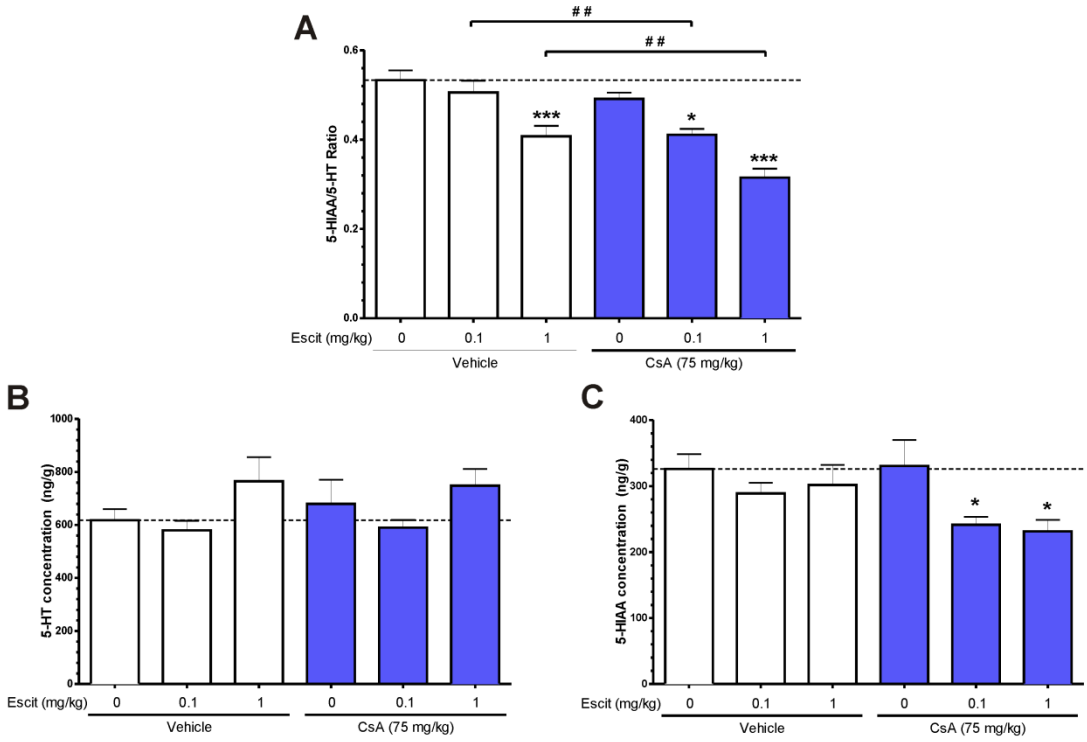

Figure 5.3: *Effect of escitalopram and cyclosporin A (CsA) on 5-HT turnover, as well as 5-HT and 5-HIAA levels, in the PFC. A Escitalopram treatment reduced 5-HT turnover (i.e. the 5-HIAA:5-HT ratio) in a dose-dependent manner, with a significant reduction at 1 mg/kg, but not at 0.1 mg/kg. CsA treatment without escitalopram did not alter 5-HT turnover. However, when both CsA and escitalopram were administered, 5-HT turnover was reduced to a greater extent than at the equivalent dose of escitalopram without CsA pre-treatment. B Treatment with escitalopram and/or CsA had no significant impact on 5-HT concentrations in the PFC. C Escitalopram or CsA treatment alone did not cause a significant change in concentrations of the 5-HT metabolite, 5-HIAA, in the PFC. However, when escitalopram and CsA treatments were combined, 5-HIAA concentrations were significantly reduced, to the same extent, in comparison to the control group at each dose of escitalopram investigated (n = 9-14 per group).*
** $p < 0.05$; *** $p < 0.001$ relative to vehicle- and saline-treated control group*
$p < 0.01$ between indicated groups in receipt of the same dose of escitalopram

5.3.2.3. *CsA pre-treatment did not augment escitalopram activity in the tail suspension test*

There was a significant difference between the groups in terms of immobility in the TST ($F(5, 58) = 9.859$, $p < 0.001$; Figure 5.4A). In vehicle-pretreated mice, escitalopram reduced the time spent immobile. Treatment with the higher (1 mg/kg) dose of escitalopram resulted in a statistically significant 40% reduction in immobility compared to the saline-treated control group ($p = 0.001$), whereas treatment with the lower (0.1 mg/kg) dose of escitalopram did not elicit a significant reduction in immobility ($p = 0.394$).

Pre-treatment with CsA had no impact on immobility in saline-treated mice ($p = 0.272$). In common with vehicle-pre-treated mice, administration of the higher dose of escitalopram significantly reduced the time spent immobile in the TST in CsA-pre-treated mice ($p < 0.001$ *vs* controls). There was no difference in immobility following administration of the higher dose of escitalopram between vehicle- and CsA-pre-treated animals ($p = 0.430$). However, in mice treated with the lower dose of escitalopram, there was a statistically significant difference between vehicle- and CsA-pre-treated animals ($p = 0.017$), with CsA pre-treatment resulting in 37% increase in the duration of immobility in the TST.

5.3.2.4. CsA treatment significantly reduced locomotor activity

Escitalopram treatment had no effect on locomotor activity ($F(1, 23) = 0.165$, $p = 0.689$; Figure 5.4B). In contrast, CsA did have a significant effect on this parameter ($F(1, 23) = 23.78$, $p < 0.001$), but there was no interaction between the two treatments ($F(1, 23) = 0.727$, $p = 0.403$). Post-hoc analysis revealed that CsA treatment significantly diminished locomotor activity, with a 40-45% reduction in the distance travelled in the activity monitoring unit, regardless of whether saline ($p = 0.01$) or escitalopram ($p = 0.005$) was subsequently administered.

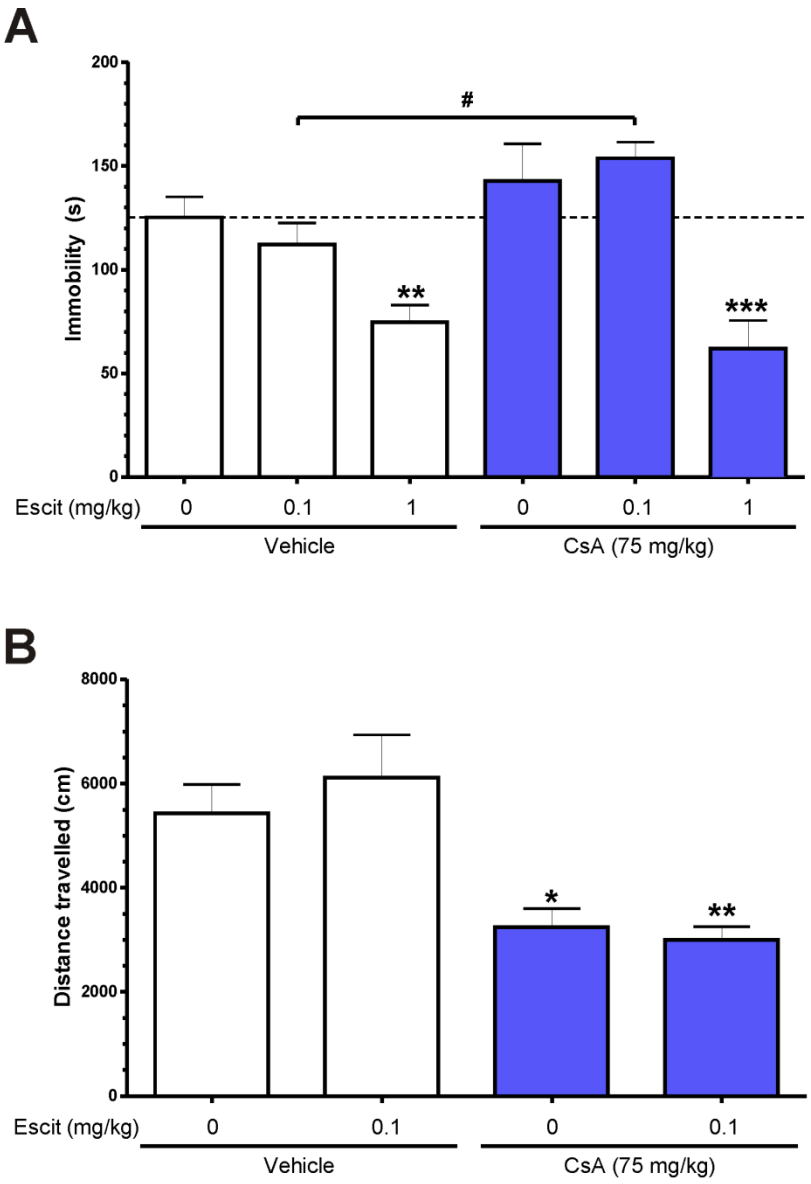

Figure 5.4: *Effect of escitalopram and cyclosporin A (CsA) on immobility in the tail suspension test (TST) and locomotor activity. A Escitalopram reduced immobility in the TST in a dose-dependent manner, with a significant 40% reduction in mice treated with 1 mg/kg but no significant reduction in mice treated with 0.1 mg/kg of escitalopram. CsA treatment, without escitalopram, had no significant impact on immobility in the TST nor did CsA augment the antidepressant-like effects of escitalopram in the TST. On the contrary, pre-treatment with CsA before the 0.1 mg/kg dose of escitalopram caused a significant increase in the time spent immobile compared to mice in receipt of the same dose of escitalopram without CsA pre-treatment (n = 9-14 per group). B CsA treatment significantly reduced locomotor activity by 40-45%, with or without escitalopram administration. Escitalopram had no effect on locomotor activity (n = 6-7 per group).*
** $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ relative to vehicle- and saline-treated control group*
$^{\#}$ $p < 0.05$ between indicated groups in receipt of the same dose of escitalopram

5.3.3. Microdialysis studies to investigate the effect of CsA pre-treatment on escitalopram-induced increases in extracellular levels of 5-HT

5.3.3.1. Pre-treatment with CsA attenuated the increase in extracellular 5-HT in the PFC in response to escitalopram administration

Pre-treatment with CsA attenuated the increase in extracellular 5-HT following escitalopram administration, relative to the vehicle-pre-treated group ($F(1, 6) = 6.899$, $p = 0.039$; Figure 5.5). In addition, there was a significant group x time interaction ($F(15, 90)$, $p < 0.001$), indicating that the time-profile of 5-HT increase differed between the groups.

In vehicle pre-treated rats, dialysate 5-HT levels rose to ~300-400% of basal levels directly after administration of escitalopram, with a maximal increase to 411% of basal levels at t = 80 min preceding a drop to ~250% at t = 240 min. In contrast, in CsA pre-treated rats, escitalopram-induced increases in dialysate 5-HT levels rose gradually and consistently to a maximum of ~223% at t = 200 min, and this increase was significantly smaller than that observed in the vehicle-treated group up to 100 min post-escitalopram administration (Figure 5.5).

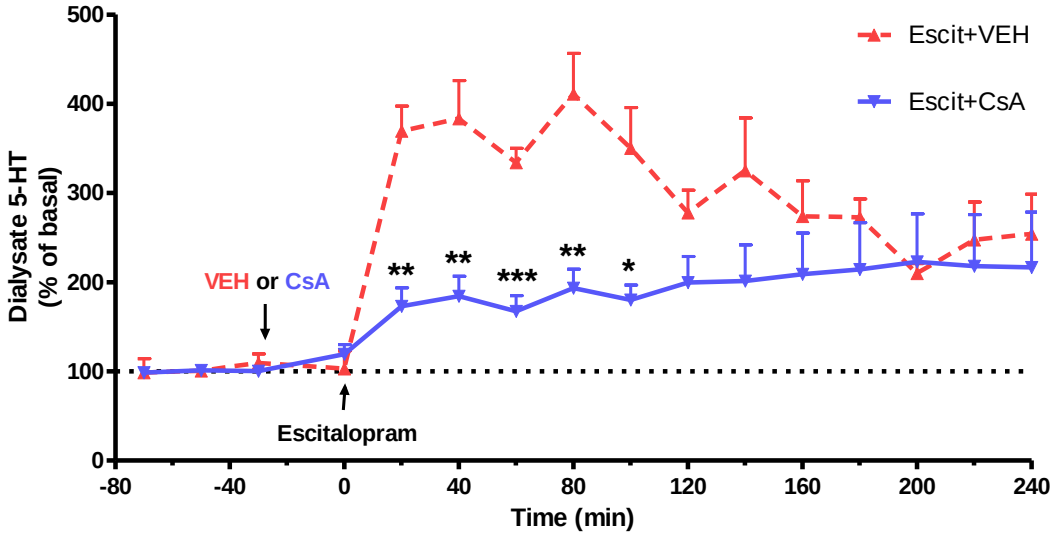

Figure 5.5: *Effect of cyclosporin A (CsA) pre-treatment on escitalopram-induced increases in extracellular 5-HT in the PFC. Compared to the vehicle-treated control group, pre-treatment with CsA transiently blunted the increases in extracellular 5-HT in response to escitalopram treatment, with significantly lower increases from 20 – 100 minutes post-escitalopram administration, inclusive (n = 4 per group).*
** p < 0.05; ** p < 0.01; *** p < 0.001 relative to vehicle-treated group*

5.4. Discussion

Increasing preclinical evidence indicates that inhibition of the drug efflux transporter P-gp at the BBB may represent a putative strategy to augment therapy with certain antidepressants by increasing their brain delivery (Ejsing and Linnet, 2005; Clarke *et al.*, 2009; O'Brien *et al.*, 2012a; O'Brien *et al.*, 2012b; O'Brien *et al.*, 2013b). However, the potential clinical consequences of this strategy in terms of increased side effects merit careful consideration. The prospect of increasing the risk of serious side effects is a particular concern in light of case studies reporting serotonin syndrome in patients treated with both 5-HT enhancing antidepressants and CsA, which is a P-gp inhibitor in addition to its primary pharmacological action as an immunosuppressant (Wong *et al.*, 2002; Lang *et al.*, 2008; Newey *et al.*, 2011). Indeed our data, using an animal model of serotonin syndrome, show for what is to our knowledge the first time that CsA can augment the behavioural effects of the SSRI escitalopram. In addition, CsA pre-treatment augmented the effect of escitalopram on 5-HT turnover in the prefrontal cortex (PFC). Intriguingly, CsA failed to enhance the effects of escitalopram in the TST, and actually blunted its neurochemical effect in terms of increases in extracellular 5-HT concentrations in the prefrontal cortex.

Serotonin syndrome is a potentially fatal adverse drug reaction, which occurs as a result of excessive 5-HT signalling following ingestion of agents which enhance serotonergic neurotransmission (Boyer and Shannon, 2005). It is characterised by clinical symptoms such as tremor, clonus, muscular hypertonicity, altered mental state and hyperthermia in patients (Boyer and Shannon, 2005). Examples of agents which may precipitate serotonin syndrome include SSRIs and other antidepressants, especially monoamine oxidase inhibitors (MAOIs), some antiemetics (e.g. ondansetron, granisetron and metoclopraminde), certain drugs of abuse (e.g. MDMA and LSD) and 5-HT precursors such as tryptophan (Boyer and Shannon, 2005; Haberzettl *et al.*, 2013). While serotonin syndrome may occur after ingestion of a single 5-HT enhancing agent, it is more commonly associated with intake of two or more serotonergic drugs with differing mechanisms of action, such as SSRIs and MAOIs (Haberzettl *et al.*, 2013). Indeed, life-threatening cases are generally only

observed where a combination of agents has been taken (Gillman, 2005). Importantly, the incidence of serotonin syndrome is thought to be rising with increased prescribing of serotonergic drugs (Thanacoody, 2012). Considering the serious and potentially fatal outcomes associated with serotonin syndrome, it is not possible to conduct human experiments to investigate drug combinations which may induce the syndrome. However, many of the symptoms of serotonin syndrome can be successfully emulated in rodent models (Haberzettl *et al.*, 2013). Thus, due to their high level of predictive validity, such rodent models of serotonin syndrome represent a valuable tool to assess drug combinations suspected of eliciting cases of serotonin syndrome in humans (Haberzettl *et al.*, 2013). In this context, the present study offers confirmation that CsA may cause or exacerbate serotonin syndrome when combined with serotonergic antidepressants, such as escitalopram, as per previous case studies reported in humans treated with CsA in combination with escitalopram, venlafaxine or sertraline (Wong *et al.*, 2002; Lang *et al.*, 2008; Newey *et al.*, 2011).

In addition to inhibiting P-gp, CsA has other pharmacological actions that may be responsible for the effects seen in the present study. For example, CsA is a potent calcineurin inhibitor and immunosuppressant. Interestingly, CsA has been reported to potentiate tremors induced by harmine, a naturally occurring plant alkaloid which inhibits the monoamine oxidase enzyme, thereby implicating the monoaminergic system in its pharmacology (Shuto *et al.*, 1998). Indeed, the present data point to pharmacodynamic interactions between CsA and escitalopram affecting serotonin transmission that may be either P-gp dependent or independent. It is worth noting that the intermediate dose of CsA used in this study (75 mg/kg) elicited a significantly milder exacerbation of the mouse model of serotonin syndrome than the other two doses used (37.5 and 150 mg/kg). The mechanism underlying this dose-dependent effect is unclear. However, pilot studies revealed that the 75 and 150 mg/kg doses of CsA resulted in similar increases in brain tissue concentrations of escitalopram (71% vs 62% increase, respectively; data not shown). These findings indicate that a P-gp independent mechanism may be at play, as the dose-dependent effect of CsA on serotonin syndrome behaviours

cannot be attributed to differences in brain levels of escitalopram. Further studies are needed to ascertain whether findings in relation to the effect of CsA in this model of serotonin syndrome occurred as a result of increased brain levels of escitalopram at key sites involved in the regulation of serotonin syndrome due to P-gp inhibition, or reflect an intrinsic effect of CsA on the serotonergic system.

We have recently demonstrated that pre-treatment with the P-gp inhibitor verapamil results in increased escitalopram levels in brain homogenates (O'Brien *et al.*, 2013b, *Chapter 4*). In the present study, the impact of pre-treatment with CsA in the same paradigm was investigated to determine if observations in relation to verapamil extend to other P-gp inhibitors. Similar increases in escitalopram brain concentrations were observed following pre-treatment with CsA as previously reported for verapamil, thereby further confirming the key role played by P-gp in the brain distribution of escitalopram.

In line with data from previous reports using different antidepressants (Connor *et al.*, 2000; Stenfors and Ross, 2002; Miura *et al.*, 2005; Miura *et al.*, 2007), escitalopram treatment was found to reduce 5-HT turnover in the PFC in a dose-dependent manner in the present study. Pre-treatment with CsA augmented this effect. Despite there being a ~5-fold difference in brain levels of escitalopram between the two groups (70 *vs* 349 ng/g, respectively), 5-HT turnover was reduced to the same extent in mice treated with CsA and the lower dose of escitalopram as those treated with the higher dose of escitalopram without CsA pre-treatment. Therefore, the augmentation of escitalopram's effect on 5-HT turnover by CsA pre-treatment cannot be fully attributed to the increased brain levels of escitalopram due to P-gp inhibition. Moreover, significant reductions in the PFC concentration of the 5-HT metabolite, 5-HIAA, were only observed when both CsA and escitalopram were administered together, regardless of brain concentrations of escitalopram.

Taken together, these observations indicate that CsA and escitalopram may work in concert to reduce 5-HT turnover by reducing 5-HT metabolism via a P-gp-independent mechanism, likely related to other pharmacological actions of CsA. Interestingly, CsA administration on its own had no effect on 5-HT turnover,

suggesting that these effects are dependent on states where alterations in 5-HT signalling are already present, as is the case following SSRI treatment.

In contrast to previous findings in relation to the P-gp inhibitor verapamil (O'Brien *et al.*, 2013b, *Chapter 4*), pre-treatment with CsA did not result in an augmentation of escitalopram's antidepressant-like activity in the TST. On the contrary, CsA pre-treatment significantly increased the time spent immobile in mice treated with the lower dose of escitalopram. The suppression of locomotor activity by CsA may preclude meaningful interpretation of its influence on behaviour in the TST, as this paradigm is sensitive to changes in locomotor activity unrelated to antidepressant-like activity. It is worth noting, however, that verapamil treatment resulted in a similar reduction in locomotion in the above-mentioned study in which it was found to augment the antidepressant-like activity of escitalopram (O'Brien *et al.*, 2013b, *Chapter 4*). Importantly, intracerebral administration of calcineurin inhibitors, including CsA, has been reported to induce depressive-like behaviour in rodent models of depression (Zhu *et al.*, 2011; Yu *et al.*, 2013), thus underlining the difficulties encountered when conducting behavioural P-gp inhibition studies using relatively non-selective inhibitors. The divergence between the effects of CsA and verapamil pre-treatment on escitalopram activity in the TST, despite comparable reductions in locomotor activity and similar increases in brain levels of escitalopram for both P-gp inhibitors, highlights the need for further studies involving more selective P-gp inhibitors, such as the CsA analogue valspodar or tariquidar, to specifically elucidate the impact of P-gp inhibition on the antidepressant-like activity of escitalopram.

In light of the behavioural data observed, where CsA increased the severity of an escitalopram-induced mouse model of serotonin syndrome but did not augment the antidepressant-like activity of escitalopram in the TST, and the combined effect of CsA and escitalopram in reducing 5-HT turnover, intracerebral microdialysis studies were undertaken to investigate the pharmacodynamic effect of co-administration of CsA and escitalopram on extracellular 5-HT. These experiments were of particular interest considering a previous microdialysis study reported that CsA administration reduced extracellular 5-HT levels in the PFC in mice by over 50%

relative to basal concentrations (Sato *et al.*, 2007). To our knowledge, the present study represents the first time that the impact of CsA administration on SSRI-induced increases in extracellular 5-HT has been investigated. These experiments revealed that CsA markedly blunted the increase in extracellular 5-HT in response to escitalopram administration. These microdialysis findings may help to explain why CsA differed to verapamil in terms of its impact on escitalopram activity in the TST, as depletion of 5-HT attenuates the response to SSRIs in this paradigm (O'Leary *et al.*, 2007). However, it must be noted that, for technical reasons, microdialysis experiments were undertaken in the rat, as opposed to mice used in behavioural studies. Thus, potential inter-species differences between rats and mice in terms of serotonergic responses to CsA need to be taken into consideration when attempting to compare results from behavioural and microdialysis experiments. Further studies, investigating the impact of CsA on serotonergic signalling in an SSRI-induced animal model of serotonin syndrome, will offer additional insight into the mechanism underlying the differential effects of CsA on behavioural responses to escitalopram. In addition, future intracerebral microdialysis experiments should be conducted to determine the effect of treatment with more selective P-gp inhibitors (Colabufo *et al.*, 2010), as opposed to CsA or verapamil, on escitalopram-induced increases in extracellular 5-HT.

5.5. Conclusion

In conclusion, the present data reveal novel, but complex, pharmacokinetic and pharmacodynamic interactions between CsA and the SSRI escitalopram. Firstly, we demonstrate that CsA pre-treatment exacerbates the severity of symptoms associated with a mouse model of serotonin syndrome. This finding, in conjunction with previously published case studies (Wong *et al.*, 2002; Lang *et al.*, 2008; Newey *et al.*, 2011), indicates that caution should be exercised if CsA is to be administered concurrently with a serotonergic antidepressant in clinical practice. The mechanism underlying this effect remains elusive. Further studies are required to determine if it is due to altered escitalopram pharmacokinetics as a result of P-gp inhibition, or as a result of a combined effect of CsA and escitalopram on serotonergic transmission related to other pharmacological actions of CsA, such as calcineurin inhibition Furthermore, a novel pharmacodynamic interaction between CsA and escitalopram was revealed, whereby these drugs acted in concert to reduce serotonin turnover in the prefrontal cortex. Finally, these studies highlight the need for further investigations using more selective pharmacological tools to specifically elucidate the effect of P-gp inhibition on the activity of antidepressants which are transported substrates of P-gp. Such experiments will serve to discriminate between pharmacokinetic effects, due to P-gp inhibition per se, and potential pharmacodynamic interactions related to alternative pharmacological effects which may be encountered when using non-specific P-gp inhibitors.

Chapter 6:

P-glycoprotein Inhibition Increases the Brain Distribution of the Antidepressant Escitalopram in the Mouse: Effects of Chronic Administration

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Abstract

Recent preclinical studies have revealed a functionally important role for the drug efflux pump P-glycoprotein (P-gp) at the blood-brain barrier (BBB) in limiting brain levels and antidepressant-like activity of certain antidepressant drugs. Specifically, acute administration of P-gp inhibitors, such as verapamil and CsA, have been shown to augment brain concentrations and functional activity of the antidepressant escitalopram in rodents. However, depression is a chronic disorder and current treatments require prolonged administration to elicit their full therapeutic effect. Thus, it is important to investigate whether acute findings in relation to P-gp inhibition translate to chronic paradigms. To this end, the present study investigates whether chronic treatment with the P-gp inhibitor verapamil and the antidepressant escitalopram results in enhanced brain distribution and antidepressant-like effects of escitalopram. Verapamil (10 mg/kg i.p.) and escitalopram (0.1 mg/kg i.p.) were administered once daily for 22 days. On the final day of treatment, brain regions and plasma were collected for analysis of cortical and plasma concentrations of escitalopram and verapamil, and to determine the hippocampal expression of genes previously reported to be altered by chronic antidepressant treatment. Verapamil treatment resulted in a greater than two-fold increase in brain levels of escitalopram, without altering plasma levels. Gene expression analysis did not reveal an augmentation of molecular responses to escitalopram treatment as a result of verapamil administration. Taken together, these data demonstrate for the first time that P-gp inhibition can yield elevated brain concentrations of an antidepressant after chronic treatment, but the functional relevance of these increased brain levels remains unclear.

6.1. Introduction

Increasing data have revealed that the multidrug efflux transporter P-glycoprotein (P-gp), expressed at the blood-brain barrier (BBB), restricts brain levels of several clinically important antidepressant drugs, thereby potentially contributing to the high prevalence of treatment failure (Uhr *et al.*, 2008; O'Brien *et al.*, 2012a; O'Brien *et al.*, 2012b; O'Brien *et al.*, 2013a). Moreover, we have recently demonstrated that acute inhibition of P-gp by verapamil enhances the behavioural effects of the antidepressant escitalopram in the tail suspension test (TST) (O'Brien *et al.*, 2013b), one of the most widely used and well-validated animal models to assess antidepressant-like activity (Cryan *et al.*, 2005a). Taken together, these findings raise the possibility that adjunctive treatment with a P-gp inhibitor may represent a potentially beneficial augmentation strategy in treatment resistant depression.

Most studies investigating the effect of P-gp on antidepressant distribution into the brain have focused on acute drug administration (O'Brien *et al.*, 2012b). While a limited number of studies have reported that brain levels of certain antidepressants are elevated in P-gp knockout mice relative to wild-type controls after subchronic (10-11 days) treatment (Grauer and Uhr, 2004; Uhr *et al.*, 2008; Karlsson *et al.*, 2011; Karlsson *et al.*, 2013), no study to date has investigated the effect of chronic P-gp inhibition on antidepressant distribution into the brain in wild-type animals, to our knowledge. This is a key consideration, as currently available antidepressants are associated with a delayed response, typically requiring chronic treatment in order to achieve their therapeutic effect in patients (Krishnan and Nestler, 2008). Moreover, certain xenobiotics are known to upregulate the expression and activity of P-gp (Miller, 2010). Indeed, the antidepressant venlafaxine, which is known to be a transported P-gp substrate (O'Brien *et al.*, 2012b), has been reported to increase P-gp function *in vitro* and *in vivo* (Ehret *et al.*, 2007; de Klerk *et al.*, 2010). Thus, even though acute P-gp inhibition can result in increased brain levels of certain antidepressants, this effect could be negated following chronic exposure due to the hypothetical counteractive upregulation of P-gp activity.

The primary goal of the present study is to determine whether chronic treatment with the P-gp inhibitor verapamil and escitalopram would increase the brain distribution of escitalopram. Recent studies, both in P-gp knockout (acute and subchronic) (Karlsson *et al.*, 2013) and wild-type (acute only) (O'Brien *et al.*, 2013b) rodents, have identified that escitalopram, a commonly prescribed selective serotonin reuptake inhibitor (SSRI) antidepressant, is a transported P-gp substrate at the BBB. In addition, putative behavioural and molecular effects of chronic treatment with a P-gp inhibitor and a P-gp substrate antidepressant are investigated. In particular, the expression of several genes involved in the regulation of monoaminergic signalling, neurogenesis, responses to stress and gene transcription and which have been reported to be sensitive to chronic antidepressant treatment was assessed (Table 6.1).

Table 6.1: Target genes selected for mRNA expression analysis

Gene name	Protein product	Function	Studies implicating gene in antidepressant response
Nr3c1	Glucocorticoid receptor (GR)	Receptor for glucocorticoids, such as corticosterone in mice	(Peiffer *et al.*, 1991; Seckl and Fink, 1992; Johansson *et al.*, 1998; Bjartmar *et al.*, 2000; Guidotti *et al.*, 2013)
Nr3c2	Mineralocorticoid receptor (MR)	Cytosolic receptor for mineralocorticoids, such as aldosterone, as well as glucocorticoids	(Brady *et al.*, 1991; Seckl and Fink, 1992; Johansson *et al.*, 1998; Bjartmar *et al.*, 2000)
Fkbp5	FK506 binding protein (FKBP5)	Immunophilin protein involved in immunoregulation and protein folding/trafficking	(Guidotti *et al.*, 2013)
Egr1	Early growth response protein 1 (EGR1; aka Zif268 or NGFI-A)	Transcription factor	(Morinobu *et al.*, 1997; Johansson *et al.*, 1998; Bjartmar *et al.*, 2000; Sillaber *et al.*, 2008)
Nr4a1	Nerve growth factor IB (NGFI-B)	Transcription factor	(Bjartmar *et al.*, 2000)
Slc6a4	Serotonin transporter (SERT)	Reuptake if 5-HT from synaptic space	(Lesch *et al.*, 1993; Lopez *et al.*, 1994; Benmansour *et al.*, 1999)
Tph2	Tryptophan hydroxylase 2 (TPH2)	Rate limiting enzyme in the synthesis of 5-HT in CNS	(Abumaria *et al.*, 2007; Shishkina *et al.*, 2007; Heydendael and Jacobson, 2009)
Htr1a	5-HT1A receptor	5-HT autoreceptor involved in regulation of 5-HT signalling	(Burnet *et al.*, 1994; Abumaria *et al.*, 2007)
Kcnk2	Trek-1	Potassium channel	(Heurteaux *et al.*, 2006)
Bdnf	Brain derived neurotrophic factor (BDNF)	Neurotrophin	(Nibuya *et al.*, 1995; Martinez-Turrillas *et al.*, 2005; Sillaber *et al.*, 2008; Alboni *et al.*, 2010)
Creb	cAMP response element-binding protein (CREB)	Transcription factor	(Nibuya *et al.*, 1996; Thome *et al.*, 2000; Blom *et al.*, 2002; Alboni *et al.*, 2010)
S100a10	p11	Involved in regulation of 5-HT signalling in brain	(Svenningsson *et al.*, 2006; Melas *et al.*, 2012)

6.2. Material and methods

6.2.1. Drugs and chemicals

Escitalopram oxalate was purchased from Discovery Fine Chemicals (Dorset, UK). Verapamil was obtained from Sigma-Aldrich (Ireland), as were all other chemicals, reagents and materials unless otherwise stated.

6.2.2. Animals

Male C57BL/6JOlaHsd mice (6-8 weeks old at the beginning of the study), purchased from Harlan Laboratories, UK, were used in this study. All animals were group-housed 4 animals per cage and maintained on a 12 hour light/dark cycle (lights on at 08:00 h) with food and water *ad libitum*. Room temperature was controlled at 22 ± 1°C. All procedures were carried out in accordance with EU directive 2010/63/EU and approved by the Animal Experimentation & Ethics Committee of University College Cork.

6.2.3. Experimental design

Verapamil (10 mg/kg i.p.) or saline were administered at least 30min before escitalopram (0.1 mg/kg i.p.) or saline each morning for 22 days (n = 9-10 per group). Body weight was measured daily to monitor for potential adverse reactions to treatment. On the penultimate day of treatment (day 21), mice were subjected to the tail suspension test to assess the effect of chronic verapamil pre-treatment on the antidepressant-like activity of escitalopram. On the final day of treatment, mice were sacrificed 40 min after escitalopram and 100 minutes post-verapamil administration. Timings were based on previous acute experiments (O'Brien *et al.*, 2013b, *Chapter 4*). Brain regions were immediately dissected out in ice-cold PBS and trunk plasma was collected. All samples were snap frozen in isopentane on dry ice, and stored at -80˚C until further processing. The dose of escitalopram was selected based on our previous study, where 0.1 mg/kg of escitalopram was found to elicit a behavioural effect in the TST only when administered in conjunction with verapamil (O'Brien *et al.*, 2013b). However, a lower dose of verapamil was used in the present study (10 mg/kg rather than 20 mg/kg) due to concerns about potential

systemic side effects that may have arisen with prolonged high dose verapamil treatment.

6.2.4. Tail suspension test

The tail suspension test (TST), one of the most widely used models for assessing antidepressant activity in rodents (Cryan *et al.*, 2005a), was carried out on the 21st day of treatment, as described previously (O'Brien *et al.*, 2013b). This facilitated investigation of the impact of pre-treatment with the P-gp inhibitor verapamil on the antidepressant-like activity of escitalopram after chronic administration of each drug. Briefly, on the day of the TST, the P-gp inhibitor verapamil (10 mg/kg i.p.) or saline was administered one hour before escitalopram (0.1 mg/kg i.p.) or saline treatment. Thirty minutes after the second injection, mice were individually suspended by the tail from a horizontal bar using adhesive tape. Six minute test sessions were recorded by video camera and the amount of time spent immobile by each animal was subsequently scored by a trained observer blind to the treatment groups.

6.2.5. Determination of escitalopram and verapamil in brain and plasma samples

Escitalopram and verapamil were extracted from cortical brain tissue and plasma using a liquid-liquid extraction technique described previously (Clarke *et al.*, 2009; O'Brien *et al.*, 2013b). Briefly, 48 µl of plasma was spiked with 2 µl of the internal standard, imipramine, to yield a final concentration of 1 µg/ml imipramine. To this imipramine-spiked plasma, 1 ml of sodium hydroxide (2 M) and 3 ml of water were added. Extraction was carried out in 7.5 ml of 1.5% isoamyl alcohol in n-heptane by vortexing for 30 seconds, followed by agitation on a mechanical shaker for 15 min and then centrifugation at 5000 rpm for 15 min at 20°C. The upper solvent layer was transferred to a tube containing 200 µl of 25 mM OPA, vortexed for 30 seconds, then agitated on a mechanical shaker for 15 min followed by centrifugation at 5000 rpm for 15 min at room temperature. Twenty microlitres of the lower aqueous phase was injected onto a HPLC system for analysis of escitalopram and verapamil, using a previously described HPLC method (O'Brien *et*

al., 2013b). Brain tissue samples were weighed prior to homogenization in 500 µl of imipramine-spiked (1 µg/ml) homogenization buffer (i.e. HPLC mobile phase). Homogenized brain tissue was centrifuged at 14000 rpm at 8°C for 15 min, and escitalopram and verapamil were extracted from 200 µl of the supernatant as described above.

6.2.6. Gene expression analysis

The hippocampal expression of twelve genes, which have been shown to be responsive to chronic antidepressant treatment or implicated in the antidepressant response (Table 6.1), was analysed using real-time quantitative polymerase chain reaction (qPCR). To our knowledge, p11, brain-derived neurotrophic factor (BDNF) and cAMP-responsive element binding protein (CREB) are the only gene targets which have been shown to be altered by chronic escitalopram treatment in the (rat) brain (Jacobsen and Mork, 2004; Alboni *et al.*, 2010; Melas *et al.*, 2012). Total RNA was extracted from hippocampal tissue using a commercially available kit (*mir*Vana™ PARIS™ Kit, Applied Biosystems, USA), as per the manufacturer's instructions. RNA purity and quantity was measured by spectrophotometric analysis (NanoDrop® ND-1000 Spectrophotometer, NanoDrop Technologies, Inc., USA). mRNA was reverse transcribed from 1 µg total RNA using a G-storm thermocycler (G-storm, Surrey, UK). PCR primers and probes were purchased from Integrated DNA Technologies (Belgium). Assays were designed for murine genes, as detailed in Table 6.1. Analysis of gene expression was performed in triplicate on 384-well plates using the LightCycler 480 System (Roche, Ireland), and the expression of each gene was normalised to that of β-actin. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expression determined from qPCR experiments (Livak and Schmittgen, 2001). Gene expression values are expressed relative to the control group.

6.2.7. Data analysis and statistical procedures

Statistical analysis of data was carried out using standard commercial software (SPSS Statistics, version 20.0.0; SPSS, Inc., Chicago, IL). The differences in brain and plasma levels of escitalopram between the two escitalopram-treated groups were

analysed using an unpaired Student's *t*-test. Similarly, verapamil concentrations in brain tissue and plasma were compared by unpaired Student's *t*-test. Differences in gene expression between the groups were analysed by two-way ANOVA, with verapamil and escitalopram as factors, and the LSD post-hoc was used to elucidate statistically significant differences between treatment groups. Differences in changes in body weight over time were analysed by two-way repeated measures ANOVA, with escitalopram and verapamil as factors. The criterion for statistical significance was $p \leq 0.05$.

6.3. Results

6.3.1. Concomitant chronic treatment with the P-gp inhibitor verapamil and escitalopram increased the brain levels of escitalopram, without affecting plasma levels

The concentrations of escitalopram in cortical brain tissue were significantly greater in mice pre-treated with the P-gp inhibitor verapamil compared to saline-pretreated mice, whereas there was no significant difference in plasma escitalopram levels (Figure 6.1A+B). Pre-treatment with verapamil resulted in a 110% increase in cortical escitalopram levels ($t(17) = -5.903$, $p < 0.001$; Figure 6.1A), but had no effect on plasma levels of escitalopram ($t(17) = -0.98$, $p = 0.341$); Figure 6.1B). Thus, the increase in brain concentrations of escitalopram can be attributed to enhanced BBB transport due to P-gp inhibition, rather than a reflection of elevated plasma concentrations.

Plasma ($t(17) = 0.274$, $p = 0.787$) and brain ($t(14) = -0.810$, $p = 0.937$) levels of verapamil were equivalent in both verapamil-treated groups, with mean (± SEM) concentrations of 390 (± 37) ng/ml in plasma and 202 (±15) ng/g in cortical tissue.

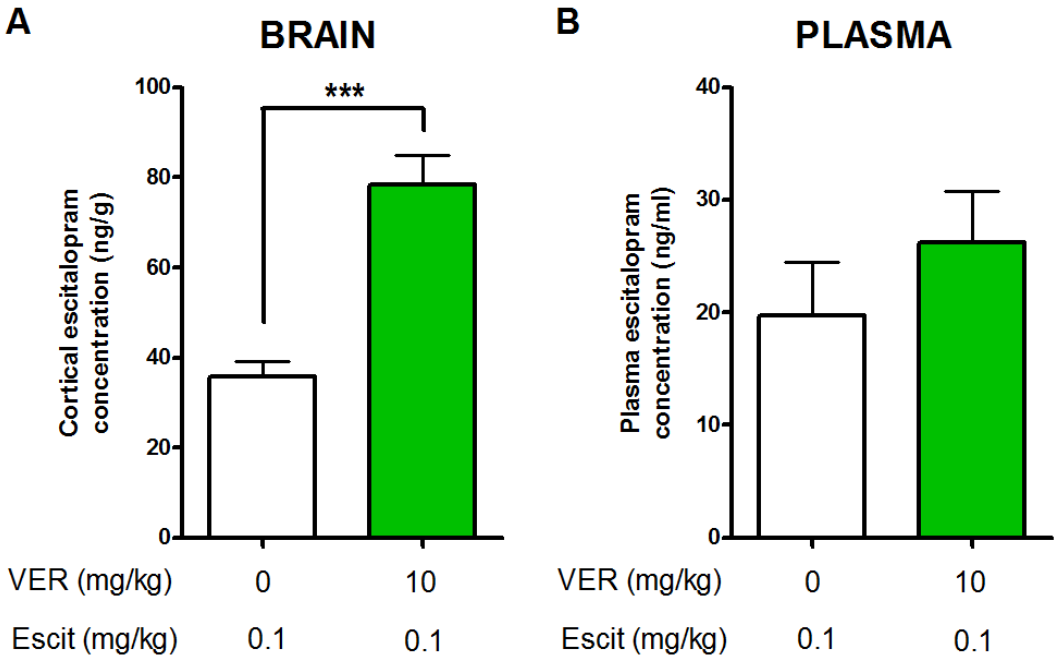

Figure 6.1: Effect of verapamil pre-treatment on brain and plasma concentrations of escitalopram after chronic administration. *A Pre-treatment with verapamil resulted in a 110% increase in concentrations of escitalopram in cortical brain tissue. B Pre-treatment with verapamil did not significantly alter plasma levels of escitalopram (n = 9-10 per group).*
**** p < 0.001 between groups*

6.3.2. Body weight

Neither drug treatment, nor a combination of both drugs, had an effect on body weight during the course of the 22 day of drug administration: escitalopram ($F(1,35) = 0.376$, $p = 0.544$); verapamil ($F(1, 35) = 0.096$, $p = 0.759$); and escitalopram x verapamil ($F(1, 35) = 0.195$, $p = 0.661$).

6.3.3. Tail suspension test

Neither escitalopram ($F(1, 35) = 0.885$, $p = 0.353$) nor verapamil ($F(1, 35) = 0.004$, $p = 0.950$) had a significant impact on the duration of immobility in the TST, nor was there an interaction between the two factors ($F(1, 35) = 1.505$, $p = 0.228$). In comparison to previously reported acute experiments performed in our laboratory

(O'Brien *et al.*, 2013b), chronic administration reduced the time spent immobile in saline-treated mice by 35% (97s *vs* 150s).

6.3.4. Gene expression analysis

For technical reasons, it was not possible to include all animals in gene expression analysis. The total numbers of samples included for each gene are listed in Table 6.2. Two-way ANOVA analysis of PCR results revealed a verapamil, escitalopram or verapamil x escitalopram effect on the expression of four of the twelve genes analysed (Table 6.2).

The only target for which a significant escitalopram x verapamil interaction was detected was the serotonin transporter (SERT). Treatment with verapamil or escitalopram individually resulted in a trend towards increased SERT mRNA expression, but this trend was reversed when both verapamil and escitalopram were administered together, resulting in a significant reduction in SERT mRNA levels compared to mice treated with escitalopram only (Figure 6.2A).

Escitalopram treatment had an effect on the expression of early growth response protein 1 (EGR1) mRNA, resulting in increased expression of this gene. Post-hoc analysis revealed that this increase was only statistically significant in mice treated with both verapamil and escitalopram. However, there was no significant interaction between the two treatments (Table 6.2; Figure 6.2B).

Verapamil treatment affected the expression of mRNA for p11 and nerve growth factor IB (NGFI-B), increasing p11 expression to a similar extent with or without escitalopram treatment (Figure 6.2C). NGFI-B mRNA expression was also increased by verapamil treatment. Similar to EGR1, post-hoc analysis revealed that this increase was only statistically significant in mice treated with both verapamil and escitalopram, but there was no significant interaction between the two treatments (Table 6.2; Figure 6.2D).

Table 6.2: *Two-way ANOVA of the effects of verapamil, escitalopram and verapamil x escitalopram interaction on mRNA expression in the hippocampus (n= 7-10).*

Target	Total n	Verapamil effect	Escitalopram effect	Verapamil x escitalopram interaction
GR	32	$F(1, 28) = 0.15$, $p = 0.904$	$F(1, 28) = 4.132$, $p = 0.052$	$F(1, 28) = 2.337$, $p = 0.138$
MR	31	$F(1, 27) = 1.530$, $p = 0.227$	$F(1, 27) = 0.004$, $p = 0.952$	$F(1, 27) = 0.059$, $p = 0.810$
FKBP5	34	$F(1, 30) = 0.810$, $p = 0.375$	$F(1, 30) = 0.669$, $p = 0.420$	$F(1, 30) = 1.385$, $p = 0.249$
EGR1	34	$F(1, 30) = 3.795$, $p = 0.061$	**$F(1, 30) = 5.854$, $p = 0.022$**	$F(1, 30) = 0.011$, $p = 0.917$
NGFI-B	31	**$F(1, 27) = 5.336$, $p = 0.029$**	$F(1, 27) = 1.838$, $p = 0.186$	$F(1, 27) = 0.389$, $p = 0.538$
SERT	33	$F(1, 29) = 0.408$, $p = 0.528$	$F(1, 29) = 0.009$, $p = 0.925$	**$F(1, 29) = 5.385$, $p = 0.028$**
TPH2	33	$F(1, 29) = 0.284$, $p = 0.598$	$F(1, 29) = 1.258$, $p = 0.271$	$F(1, 29) = 3.342$, $p = 0.078$
5-HT1A	33	$F(1, 29) = 0.008$, $p = 0.930$	$F(1, 29) = 1.091$, $p = 0.305$	$F(1, 29) = 0.414$, $p = 0.525$
TREK-1	33	$F(1, 29) = 1.568$, $p = 0.221$	$F(1, 29) = 0.091$, $p = 0.765$	$F(1, 29) = 2.216$, $p = 0.147$
BDNF	34	$F(1, 30) = 0.000$, $p = 0.990$	$F(1, 30) = 0.918$, $p = 0.346$	$F(1, 30) = 0.062$, $p = 0.805$
CREB	32	$F(1, 28) = 2.163$, $p = 0.153$	$F(1, 28) = 0.008$, $p = 0.930$	$F(1, 28) = 0.477$, $p = 0.496$
p11	34	**$F(1, 30) = 10.182$, $p = 0.003$**	$F(1, 30) = 0.076$, $p = 0.785$	$F(1, 30) = 0.127$, $p = 0.724$
Bold font denotes statistically significant effect				

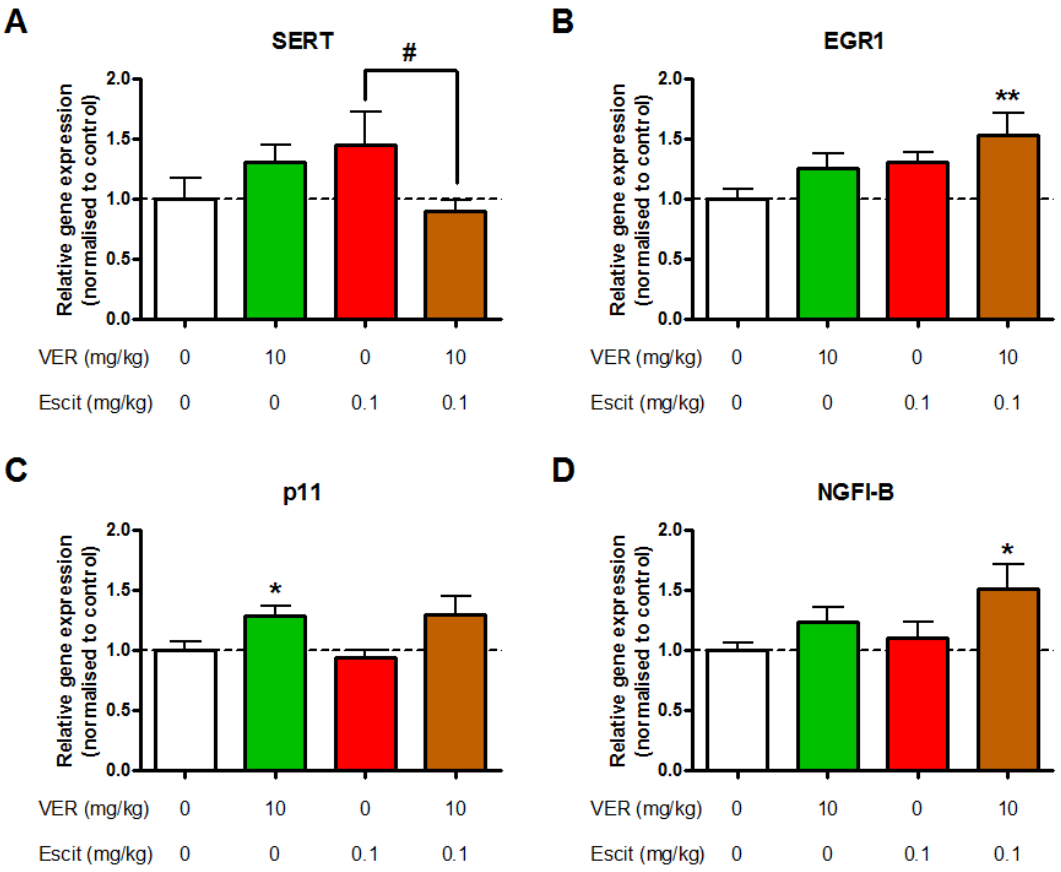

Figure 6.2: Relative expression of genes for which significant effects were observed. *A. A significant interaction between verapamil and escitalopram was observed for SERT expression. SERT mRNA levels were significantly lower in mice treated with both verapamil and escitalopram than those treated with escitalopram only. B. Escitaloram treatment was found to exert a significant effect on EGR1 mRNA expression. The increase in EGR1 mRNA levels, compared to the control group, was only statistically significant in mice treated with both verapamil and escitalopram. However, there was no significant interaction between the two treatments. C. Verapamil exerted a significant effect on p11 expression, resulting in a significant increase in mice treated with verapamil only compared to control mice, and a trend towards increased expression in mice treated with both verapamil and escitalopram (p = 0.054). D. Verapamil also exerted a significant effect on NGFI-B expression. A statistically significant elevation in NGFI-B mRNA was only observed in mice treated with both verapamil and escitalopram, but there was no interaction between the two treatments (n = 7-10 per group).*
** p < 0.05; ** p < 0.01 compared to control group*
 # p < 0.05 between the indicated groups

6.4. Discussion

Recent evidence from preclinical studies has highlighted an important role for the efflux transporter P-gp at the BBB in limiting the brain distribution of several antidepressants (O'Brien *et al.*, 2012b). Here, we demonstrate, for what is to our knowledge the first time, that chronic treatment with a P-gp inhibitor and a P-gp substrate antidepressant results in increased brain levels of the antidepressant drug. We recently demonstrated that acute pre-treatment with the P-gp inhibitor verapamil augmented the brain concentrations and functional activity of the SSRI escitalopram in mice. However, prior to the present study, it remained unclear if such findings would translate to chronic treatment paradigms. Considering that antidepressant drugs are administered chronically in clinical practice, this is an important consideration, especially given that the antidepressant venlafaxine has been reported to induce P-gp *in vitro* and *in vivo* (Ehret *et al.*, 2007; de Klerk *et al.*, 2010). Thus, the present results indicate that P-gp inhibition may represent a promising strategy to augment the brain delivery of certain antidepressants during chronic treatment.

Verapamil, the P-gp inhibitor used in the present study, is a clinically used calcium channel blocker, indicated for the treatment of cardiovascular conditions such as angina, supraventricular tachycardia and essential hypertension. At higher doses, which it is thought may be required to inhibit P-gp due to its lack of specificity and relatively low potency, verapamil can elicit toxicity, characterized by symptoms such as hypotension and bradycardia (DeWitt and Waksman, 2004). In the present study, in which verapamil was found to inhibit P-gp, no overt signs of toxicity were detected during daily observations or in terms of alterations in body weight. Furthermore, the plasma concentrations of verapamil measured in the present study (390 ng/ml) are within the clinical therapeutic range (50 - 500 ng/ml) (Regenthal *et al.*, 1999). Notwithstanding the potential for verapamil to inhibit P-gp, it is likely that its other pharmacological actions, including cardiovascular effects and inhibition of cytochrome P450 enzymes, would preclude its widespread use as a P-gp inhibitor in clinical practice. Several more specific second and third generation P-gp inhibitors have been developed which may be more appropriate

for this purpose (Colabufo *et al.*, 2010). Nonetheless, verapamil represents a valuable pharmacological tool to investigate the effects of P-gp inhibition on drug distribution in preclinical studies.

Despite observing a greater than 2-fold increase in escitalopram concentrations in cortical tissue in mice treated with the P-gp inhibitor verapamil, no enhancement of behavioural or gene expression responses to chronic escitalopram treatment was detected. Indeed, chronic treatment with escitalopram elicited no robust effect on the behavioural or molecular parameters investigated in the present study. This is perhaps not too surprising, given that we chose a relatively low dose (0.1 mg/kg) of escitalopram so as to be able to unmask any augmentation effects of verapamil.

Nonetheless, in previous acute studies, we had observed a behavioural effect in the TST at this dose of escitalopram, but only when mice were pre-treated with verapamil (O'Brien *et al.*, 2013b, *Chapter 4*). No such effect was evident here after chronic administration of both drugs. However, the results from the TST are inconclusive: when compared to our previous acute experiments (cf. O'Brien *et al.*, 2013b, *Chapter 4*), the baseline immobility in the control group was reduced by 35%. This may have resulted in a floor effect, thereby potentially obscuring any putative behavioural impact of the increased brain concentrations of escitalopram due to P-gp inhibition. Although there have been a limited number of reports of the TST being responsive to chronic antidepressant administration, it is predominantly used to assess behavioural responses to acute antidepressant treatment (Cryan *et al.*, 2005a). Interestingly, studies which have yielded positive results in the TST following chronic antidepressant administration have generally involved quite high doses of the antidepressant, for example 20 mg/kg of fluoxetine (Ukai *et al.*, 1998). Hence, increasing the dose of escitalopram may represent a viable strategy to overcome the floor effect seen here. Future investigations of the effect of P-gp inhibition on antidepressant-like activity should make use of other paradigms, which are responsive to chronic antidepressant treatment, such as the social defeat stress or learned helplessness models (O'Leary and Cryan, 2013).

Similar to our behavioural studies, gene expression analysis did not reveal an augmentation of molecular responses to escitalopram administration in verapamil pre-treated mice. However, reports of alterations in mRNA expression in response to antidepressant treatment are often inconsistent, and may not be universal among different antidepressants, as evidenced by the variable findings in relation to BDNF expression (Tardito *et al.*, 2006; Groves, 2007). Thus, genes which have been reported to be altered by treatment with other antidepressants may not be influenced by escitalopram administration. To our knowledge, p11, BDNF and CREB are the only gene targets which have been shown to be affected by chronic escitalopram treatment (Jacobsen and Mork, 2004; Alboni *et al.*, 2010; Melas *et al.*, 2012). However, the expression of these genes was not altered by escitalopram treatment in the present study, further underlining the inconsistency of the effects of antidepressant treatment on mRNA expression. Moreover, our finding that chronic escitalopram treatment exerted a significant effect on mRNA levels of the transcription factor EGR1 is the first time that this has been reported, to our knowledge. Interestingly, the combination of verapamil and escitalopram treatment, but neither in isolation, resulted in a statistically significant increase in EGR1 mRNA expression relative to control mice. Similar findings were observed in relation to another transcription factor, NGFI-B. While there was not a significant interaction between escitalopram and verapamil treatment for either of these genes, indicating that this could merely reflect an additive effect of the two treatments, these targets warrant further investigation in future studies.

It is also worth noting that antidepressant-induced changes in gene expression can be dependent on the brain region analysed and the antidepressant dose used (Tardito *et al.*, 2006). In relation to the dose, the drug administration regimen used in the present study yielded plasma concentrations of escitalopram (23 ng/ml) within the therapeutic range (15 – 80 ng/ml) (Baumann *et al.*, 2004), albeit at the lower end of that range. However, most studies which have reported significant alterations in mRNA expression following antidepressant treatment have involved the administration of much higher doses of the antidepressant (10 mg/kg escitalopram daily, for example (Jacobsen and Mork, 2004; Alboni *et al.*, 2010)).

Thus, the doses of escitalopram used in the present study may have been insufficient to reproduce previously reported changes in mRNA expression. Furthermore, it is possible that there may have been functionally relevant alterations in protein expression, activation or cellular localization in response to antidepressant treatment, without changes in mRNA levels. Future studies should investigate these possibilities.

Even though our analysis did not reveal any augmentation of escitalopram's effects on mRNA expression by verapamil administration, a statistically significant interaction between the two treatments was observed in terms of serotonin transporter (SERT) mRNA levels. Interestingly, this effect constituted an attenuation of a trend towards increased SERT mRNA levels following verapamil or escitalopram on their own. It is important to note that, while studies have shown that SERT mRNA expression can be altered in response to antidepressant treatment, these findings have been somewhat inconsistent. SERT mRNA levels have been variously reported to be increased, decreased or not affected by chronic antidepressant treatment (Lesch *et al.*, 1993; Lopez *et al.*, 1994; Benmansour *et al.*, 1999; Abumaria *et al.*, 2007), indicating that the effect of antidepressant drugs on SERT expression is complex and variable.

Due to the inconclusive nature of our findings in relation to the effect of chronic P-gp inhibition on behavioural and molecular responses to antidepressant treatment, further studies are required to investigate this question. Such future studies should ideally utilise more specific inhibitors of P-gp, thereby limiting the contribution of P-gp independent factors to alterations in gene expression, and helping to specifically elucidate antidepressant-related changes. For example, two-way ANOVA analysis revealed that verapamil itself exerted a significant effect on the expression of mRNA for NGFI-B and p11, thus making it difficult to identify any potential influence of P-gp inhibition on putative escitalopram effects in relation to these targets. In addition, future studies should use different dosing regimens of escitalopram and investigate other parameters, such as protein expression, rather than focusing on mRNA expression exclusively.

In conclusion, the present study demonstrates that inhibition of P-gp results in elevated brain levels of an antidepressant drug in a chronic drug administration paradigm. This finding indicates that P-gp inhibition may represent a viable strategy to enhance the brain distribution of P-gp substrate antidepressants after repeated dosing. In addition, these data reveal that chronic escitalopram administration can influence mRNA expression of the transcription factor EGR1. Further studies are required to fully elucidate the functional consequences, if any, of increasing the brain levels of an antidepressant drug by P-gp inhibition on a prolonged basis.

Chapter 7:
General Discussion

7.1. Overview and Summary

In this thesis, we have demonstrated that the drug efflux pump P-glycoprotein (P-gp) restricts the blood-brain barrier (BBB) transport of the antidepressants imipramine and escitalopram in rodents. Inhibition of P-gp, by either verapamil or cyclosporin A (CsA), resulted in a significant increase in brain concentrations of both of these antidepressants following acute administration. Further studies revealed that the acute effect of P-gp inhibition on the brain distribution of escitalopram is also relevant to chronic treatment scenarios. Moreover, we have shown that both imipramine and escitalopram are transported substrates of human P-gp, using an *in vitro* bidirectional transport approach, indicating that our findings in rodents may also apply to humans. In contrast, we found that the antidepressants amitriptyline, duloxetine, fluoxetine and mirtazapine are not transported substrates of human P-gp *in vitro*, demonstrating that it is necessary to assess the P-gp substrate status of antidepressant drugs on a case-by-case basis. Furthermore, we investigated functional consequences of increasing brain levels of antidepressants by modulating P-gp function, demonstrating that P-gp inhibition by verapamil augmented the antidepressant-like activity of escitalopram, as measured in the tail suspension test (TST) in mice, and that CsA exacerbated behavioural responses to escitalopram in a mouse model of serotonin syndrome, indicating that adjunctive P-gp inhibition may potentially increase the risk of serious adverse reactions to antidepressant treatment.

It should also be noted that, unlike verapamil, CsA pre-treatment did not augment the effect of escitalopram in the TST, despite similar increases in brain concentrations of escitalopram. Thus, further studies are needed to fully characterise the functional relevance of P-gp inhibition on the effects of antidepressant treatment.

Nonetheless, the studies outlined in this thesis advance our understanding of the role of P-gp in the pharmacokinetics and pharmacodynamics of antidepressant drugs, and support the concept that modulation of P-gp could represent a

beneficial therapeutic approach to augment antidepressant treatment, especially in cases of treatment resistant depression (TRD).

7.2. The need for more effective strategies to improve response to antidepressant treatment

With the possible exception of agomelatine, currently available antidepressant medications act primarily by modulating monoaminergic function in the brain (Section 1.4.2). While these antidepressant drugs fail to achieve an adequate response in up to 60% of patients, and require prolonged treatment to elicit a clinical effect in those who do respond, they remain the best therapeutic option available at present (Hollon *et al.*, 2002; Fava, 2003). Much effort has been directed at attempts to unravel the neurobiological mechanisms of depression beyond the monoamine hypothesis, in the hope that a greater understanding of the pathophysiology the disorder may reveal novel therapeutic targets, resulting in more rapid and more efficacious antidepressant treatments (Krishnan and Nestler, 2008). Although this research has led to the identification of numerous systems, in addition to the monoaminergic system, which contribute to the aetiology of depression, a 'universal theory of depression' has not been elucidated. Moreover, this work has not resulted in the successful development of more efficacious antidepressant drugs (Krishnan and Nestler, 2008). Indeed, such is the lack of success in the development of novel antidepressant treatments that it is widely acknowledged that those in use in the present day are no more effective in relieving the devastating symptomatology of depression than imipramine, which was serendipitously discovered over 50 years ago. However, recent findings in relation to glutamatergic involvement in depression offer much promise, and the results of clinical trials focused on drugs targeting this mechanism are awaited with interest (Burgdorf *et al.*, 2013).

In the absence of more effective drugs targeting novel mechanisms, clinicians endeavour to maximise the therapeutic benefit attainable with currently available antidepressant drugs (Connolly and Thase, 2011). Several pharmacological options are available in cases of treatment failure, including dose optimisation, switching to another antidepressant or augmentation of antidepressant treatment with another

agent, in addition to other, non-pharmacological, interventions, such as electroconvulsive therapy (Connolly and Thase, 2011). The recent STAR*D study found that applying these (pharmacological) options in a stepwise fashion, according to a defined algorithm, resulted in remission in approximately 70% of patients, but that it required repeated trials of sufficiently sustained, vigorously dosed antidepressant therapy to achieve this response (Gaynes *et al.*, 2008). Furthermore, the authors concluded that "should the first treatment fail, either switching treatment or augmenting the current treatment" are reasonable options (Gaynes *et al.*, 2008). However, the STAR*D study was not placebo controlled, and it is important to note that the evidence base supporting the efficacy of currently used augmentation strategies is far from convincing, especially in the context of the more modern and most commonly used SSRI antidepressant drugs (Connolly and Thase, 2011). Moreover, the mechanisms of action of these augmentation agents are poorly defined. Thus, there remains scope to develop new strategies to increase the efficacy of currently available antidepressants, in addition to a clear need for more efficacious drugs which exploit novel mechanisms for the treatment of depression.

In this context, targeted inhibition of P-gp efflux represents a promising alternative to currently accepted augmentation strategies. The research outlined in this thesis builds on previous studies which initially revealed the key role played by P-gp in limiting the BBB transport of several antidepressants. Prior to the research conducted in this thesis, with the exception of three studies (Ejsing and Linnet, 2005; Ejsing *et al.*, 2006; Clarke *et al.*, 2009), the existing literature demonstrating that P-gp limited brain levels of antidepressant drugs had focused exclusively on investigations involving P-gp knockout mice.

7.3. Limitations of studies in P-gp knockout mice

While P-gp knockout mice without doubt represent an invaluable investigational tool in determining the role of P-gp in drug distribution, there are several limitations associated with studies involving their use.

Firstly, studies in P-gp knockout mice do not offer information regarding the capacity to target P-gp as a strategy to increase brain concentrations of its transported substrates. Indeed, a previous study has reported a failure to reproduce observations in P-gp knockout mice by pharmacological inhibition of P-gp (Ejsing and Linnet, 2005). Similarly, this limitation extends to studies involving the effect of P-gp on antidepressant distribution into the brain following chronic treatment. Given that the expression and activity of ABC transporters can be modulated by administration of certain xenobiotics, including the P-gp substrate antidepressant venlafaxine (Ehret *et al.*, 2007; de Klerk *et al.*, 2010), it is plausible that chronic treatment with a P-gp inhibitor and an antidepressant could result in an upregulation of P-gp activity. Considering that P-gp knockout mice do not express P-gp at the BBB, the effect of chronic drug treatment on the expression and function of P-gp, and by extension the BBB transport of P-gp substrate drugs, cannot be investigated in P-gp knockout mice. Moreover, P-gp knockout mice are developmentally different to their wild-type counterparts; there is evidence to suggest that the expression and function of other ABC efflux transporters, such as breast cancer resistance protein (BCRP), is upregulated in the absence of P-gp (Cisternino *et al.*, 2004). Given the extensive overlap in substrate specificity between the different transporters in the ABC superfamily (Sharom, 2008), this is a potentially crucial confounding factor.

The apparent discrepancy in findings between studies utilising single knockout *abcb1a*$^{(-/-)}$ mice (i.e. those lacking the *abcb1a* homologue only) and double knockout *abcb1ab*$^{(-/-)}$ mice is another important consideration in relation to P-gp knockout mice. For reasons which remain unclear, discrepant findings about the P-gp substrate status of several glucocorticoids, as well as the antidepressants fluoxetine and amitriptyline, have been reported in studies using the two strains, thereby leading to uncertainty regarding the role of P-gp in their brain distribution (Uhr *et al.*, 2000; Doran *et al.*, 2005; Uhr *et al.*, 2007; Mason *et al.*, 2012). Given that *abcb1a* is thought to be the predominant isoform of P-gp at the BBB in rodents, it is difficult to rationalise these findings.

Another limitation of many of the previously existing studies regarding the role of P-gp in the brain distribution of antidepressants is the focus on brain concentrations at a particular, individual timepoint after drug administration. As demonstrated in studies by Uhr and colleagues in relation to amitriptyline, the time of sampling can influence the results obtained (Uhr *et al.*, 2007). Thus, potentially significant differences between P-gp knockout and wild-type mice may not have been detected in some studies due to the sampling time.

Finally, P-gp deficient mice have been reported to behave abnormally in relation to their wild-type comparators in commonly used animal models of antidepressant activity, such as the forced swim test (FST) and the tail suspension test (TST) (Schoenfelder *et al.*, 2012). Thus, it may not be possible to assess the impact of P-gp knockout on responses to antidepressant treatment using these commonly used and well-validated models.

Due to these limitations, there remained a need for further studies in wild-type animals to further investigate the potential for pharmacological manipulation of P-gp to modulate antidepressant pharmacokinetics and pharmacodynamics.

7.4. An adaptable, integrated intracerebral microdialysis approach to assess drug transport across the BBB *in vivo*

To allow us to address some of the issues highlighted in the previous section, we developed an integrated intracerebral microdialysis technique to facilitate the assessment drug transport across the BBB *in vivo* in the conscious, freely moving rat. This approach involved catheterization of the jugular vein and carotid artery, and implantation of a microdialysis probe into the brain. While technically challenging, this technique offered several advantages over more simplistic methods of assessing BBB transport.

The advantages of the intracerebral microdialysis have already been outlined in Section 1.2.5.4, and are summarised in Table 7.1. Jugular vein catheterization facilitated intravenous drug administration without a need to handle or restrain the animal, thereby minimising pharmacokinetic variability by eliminating the drug absorption step associated with other routes of administration (such as

intraperitoneal, subcutaneous or oral), while also avoiding stress associated with handling or restraint. Catheterization of the carotid artery in this model facilitated repeated blood sampling from the animal while simultaneously monitoring fluctuations in free drug concentrations in the extracellular space in the brain. Again, using the catheterization approach, it was possible to do this without a need to handle or restrain the animal. The simultaneous and repeated sampling from the carotid artery and microdialysis probe allowed us to measure drug concentrations in the blood and the brain with a high level of temporal resolution. Thus, it was uniquely possible to determine the effect of P-gp inhibition on the BBB transport of antidepressant drugs over time by adopting this approach.

Table 7.1: Advantages and limitations of intracerebral microdialysis technique (de Lange *et al.*, 2000; Anderzhanova and Wotjak, 2013)

Advantages
Facilitates the monitoring of changes in the extracellular concentration of various compounds of interest in conscious freely animals
Only free, unbound, physiologically and pharmacologically active concentrations of the compound are monitored
Amenable to studies investigating neurotransmission as well as drug pharmacokinetics
Minimal disruption of tissue homeostasis
Efficient spatial and temporal resolution
Samples do not require purification or work-up prior to analysis
Limitations
Physiological stress associated with the invasive surgical procedure required
Physical damage caused by implantation of the microdialysis probe
Requirement for highly sensitive analytical assays to facilitate determination of compounds in small volume, low concentration samples
Challenges in achieving quantitative analysis of actual brain ECF concentrations
Potential changes in probe recovery due to manipulations which dramatically change the extracellular concentrations of the analyte of interest
Unsuitable for highly lipophilic compounds
Temporal resolution is restricted to several minutes (e.g. 20 min), thereby unsuitable for the measurement of rapid changes in analyte concentration

Using this technique, we demonstrated that P-gp inhibition results in increased brain levels of the antidepressants imipramine and escitalopram. Furthermore, we were able to investigate the effect of pre-treatment with the P-gp inhibitor CsA on

increases in extracellular serotonin in response to escitalopram treatment, thereby offering unique insight into the pharmacodynamic effects of this drug combination.

The possibility to administer a drug as a continuous intravenous infusion, due to the presence of the indwelling jugular vein catheter, allowed us to achieve and maintain steady state concentrations of escitalopram in plasma and brain tissue. This proved to be a particularly useful strategy when investigating the influence of P-gp inhibition on drug transport across the BBB, as demonstrated in *Chapter 4*, where a striking increase in brain levels of escitalopram were observed immediately after administration of the P-gp inhibitor CsA (O'Brien *et al.*, 2013b). Furthermore, this approach enabled us to cease the experiment at a point when the elevation in brain levels was maximal, thereby facilitating confirmation of microdialysis findings by measuring escitalopram concentrations in brain tissue harvested at termination of the experiment. This was not possible in our earlier studies involving a one-off, bolus administration of imipramine; brain levels of imipramine, as measured by microdialysis, were no longer different between the groups at the end of the experiment. Thus, it is advisable that future studies investigating the effect of drug efflux transporters on BBB transport should avail of the steady state approach. Indeed, recent work in our lab has adapted this strategy to investigate the effect of OAT3 inhibition on the BBB transport of bumetanide, with promising results to date (Donovan, O'Brien *et al.,* unpublished data).

One of the major challenges in successfully implementing the intracerebral microdialysis technique is the requirement to develop a high sensitivity analytical method to facilitate detection of the analyte of interest. For this purpose, we were able to successfully adapt methods described in the literature for the antidepressant drugs investigated in the present study. In addition, it was possible to modify our microdialysis technique to measure extracellular serotonin concentrations, both at basal levels and in response to escitalopram treatment, thereby facilitating investigation of the effect pre-treatment with the P-gp inhibitor CsA on escitalopram-induced increases in extracellular serotonin. The analytical challenge presented by microdialysis studies highlights another advantage of conducting drug transport experiments at steady state with continuous intravenous

drug infusion. When using this approach, drug concentrations can be maintained at higher, and therefore more readily detectable, levels compared to bolus administration studies. After bolus administration, drug concentrations rapidly decline to low levels from an initial peak, thereby potentially presenting an insurmountable analytical challenge. Thus, by using the steady state approach, our integrated intracerebral microdialysis technique could be applied to drugs for which highly sensitive analytical assays do not exist. The numerous applications to which this integrated intracerebral microdialysis model has already been successfully employed highlight its flexibility and utility, and indicate that it may be possible to extend its use to address other research questions.

7.5. The importance of *in vitro* studies to identify drugs which are transported substrates of human P-gp

Despite the abundance of preclinical evidence demonstrating that P-gp limits the brain distribution of antidepressants in rodents, the existing data from *in vitro* studies investigating interactions between antidepressants and human P-gp were inconclusive (O'Brien *et al.*, 2012b; *Chapter 1*). While several studies reported significant interactions between certain antidepressants and human P-gp using the ATPase assay, the relevance of these findings to drug transport is unclear. In contrast, the *in vitro* bidirectional transport approach represents the 'gold standard' approach for the identification of transported substrates of P-gp (Balimane *et al.*, 2006).

However, bidirectional transport studies investigating if antidepressant drugs are transported substrates of human P-gp have reported inconsistent, but generally negative, findings. Confident interpretation of the results from these studies is difficult, however, mostly due to methodological limitations of the two studies which have provided most of the data in this regard (Mahar Doan *et al.*, 2002; Feng *et al.*, 2008). For example, one of these studies, which investigated the effects of human P-gp on the permeability of 39 compounds in the MDCKII-MDR1 cell line, found that none of the seven antidepressants included in the study were transported substrates of P-gp (Feng *et al.*, 2008). Presumably to facilitate high throughput screening of the large number of compounds included in the study, the

effect of P-gp on drug transport was only determined at a single, 5-hour time-point. While this approach was sufficient to identify very high affinity substrates of P-gp, such as ritonavir, it may have lacked the sensitivity to detect less avid substrates. Indeed, the transport ratio for verapamil, a well-established P-gp substrate, in addition to being an inhibitor of P-gp, was marginally above the threshold for identification of a transported P-gp substrate in this study (Feng *et al.*, 2008). Another study reported that 10 of 11 antidepressants investigated were not transported substrates of P-gp (Mahar Doan *et al.*, 2002). However, the donor concentration used in this work was twice the recommended upper limit of 5 µM for these types of studies and samples were only taken at single timepoint one hour after commencing the experiment (see Balimane *et al.*, 2006, for a discussion of methodological considerations). Similarly, two of the three *in vitro* bidirectional transport studies which have reportedly identified antidepressant drugs as transported substrates of P-gp were carried out in the Caco-2 cell line, without conducting additional experiments to confirm that P-gp was the transporter responsible for the net efflux effect observed (Faassen *et al.*, 2003; El Ela *et al.*, 2004). Thus, there was a lack of definitive and reliable data pertaining to the transport of antidepressants by human P-gp. This was a major limitation when attempting to evaluate the relevance of findings from rodent studies to humans in receipt of antidepressant treatment, considering reports of species differences in P-gp specificity and activity (Yamazaki *et al.*, 2001; Katoh *et al.*, 2006; Baltes *et al.*, 2007; Syvanen *et al.*, 2009). It was in this context that we established and utilised an *in vitro* bidirectional transport model to assess if various antidepressants are transported substrates of P-gp.

In the *in vitro* model employed in our work (*Chapters 3 and 4*), drug transport was measured at several time points throughout the experiments, rather than taking a single measurement at the end, thus enabling us to determine the rate of transport more accurately (Youdim *et al.*, 2003). Moreover, cellular retention of the compound, an often overlooked factor when calculating permeability values based on experimental observations, was accounted for in our studies (Youdim *et al.*, 2003). Using this approach, we determined that P-gp substantially limited the

ability of escitalopram to traverse the cell monolayer, while it also restricted imipramine permeability, albeit to a lesser extent. Moreover, we found that P-gp exerted no effect on the permeability of amitriptyline, duloxetine, fluoxetine or mirtazapine. Thus, the *in vitro* model was sensitive enough to discriminate between antidepressants which were subjected to P-gp mediated efflux and those which were not. The data from these studies suggest that P-gp may play a role in the brain distribution of escitalopram and imipramine in humans, but is unlikely to affect the BBB transport of amitriptyline, duloxetine, fluoxetine or mirtazapine. This information in regard to amitriptyline and fluoxetine is particularly interesting, as there had been ambiguity about the role of P-gp in their brain distribution based on somewhat inconsistent findings from studies involving P-gp knockout mice (Uhr *et al.*, 2000; Doran *et al.*, 2005; Uhr *et al.*, 2007).

Knowing which antidepressants are transported substrates of P-gp would be essential in rationally designing any potential future clinical studies with the aim of evaluating P-gp inhibition as an antidepressant augmentation strategy. This knowledge will help to avoid potential flaws in study design similar to those which may have contributed to the failure of large scale clinical trials investigating P-gp inhibitors as adjunctive agents in oncology (as outlined in Section 1.3.5). Similarly, information regarding the transport of antidepressants by human P-gp is important when planning or critically evaluating pharmacogenetic studies investigating potential links between SNPs in *ABCB1* and antidepressant response.

7.6. Determining the functional relevance of increasing brain levels of antidepressant drugs by P-gp inhibition: behavioural studies

While several studies had reported that the brain pharmacokinetics of certain antidepressants were enhanced by P-gp inhibition or P-gp knockout, no published study had investigated the functional effect of increased brain distribution of antidepressants in a relevant animal model of antidepressant activity. Indeed, it had been proposed that a 4-fold increase in brain levels of a drug in P-gp knockout mice relative to wild-type controls was required in order to consider P-gp efflux to be functionally relevant (Feng *et al.*, 2008); the greatest increase reported for an

antidepressant to date is a 3.6-fold increase (O'Brien *et al.*, 2012b). Thus, it was unclear whether the differences in brain pharmacokinetics of antidepressant drugs due to P-gp inhibition or P-gp knockout would result in a meaningful difference in the pharmacodynamic effects of the antidepressant.

One previous study had reported altered behaviour in the open field in P-gp knockout mice, compared to wild-type controls, when treated with the P-gp substrate antidepressant venlafaxine (Karlsson *et al.*, 2011). While this finding was certainly interesting, as it revealed a differential response to antidepressant treatment between the two strains, its relevance to the antidepressant effects of venlafaxine were unclear. Thus, there remained a major impetus to carry out further experiments to elucidate the impact of a reduction in P-gp function, either due to pharmacological intervention or genetic ablation, on the pharmacodynamic activity of P-gp substrate antidepressants. The related, but subtly different, forced swim test (FST) and tail suspension test (TST) are two of the most widely used and well validated rodent models for assessing antidepressant-like activity (Cryan and Holmes, 2005), and would therefore represent ideal paradigms in which to address this issue. However, it may not be possible to conduct such experiments in the P-gp knockout mice, as they have been reported to display aberrant behaviour in these tests, with significantly lower immobility in the FST and significantly greater immobility in the TST, relative to their wild-type counterparts (Schoenfelder *et al.*, 2012). Therefore, P-gp knockout mice may not respond normally to antidepressant drugs in these tests; indeed, this seems to be the case (Schmitt *et al.*, 2009).

In light of the aberrant responses of P-gp knockout mice in these commonly used animal models of antidepressant activity, it was necessary to conduct pharmacodynamic studies involving P-gp inhibition in wild-type animals to assess the functional relevance of increased brain levels of antidepressants as a result of reduced P-gp function. Our experiment demonstrating that pre-treatment with verapamil augmented the antidepressant-like activity of escitalopram in the TST was the first such study (*Chapter 4*). However, subsequent investigations using an alternative P-gp inhibitor, CsA, yielded conflicting results (*Chapter 5*). CsA itself was found to exert an effect on the serotonergic system, as demonstrated by its

attenuating effect on escitalopram-induced increased in extracellular serotonin and its effect on serotonin turnover when administered in combination with escitalopram. In addition, it significantly reduced locomotor activity in the open field, which may explain why it did not augment the effect of escitalopram in the TST. Thus, it is difficult to draw any firm conclusions from pharmacodynamic studies involving CsA. Further experiments, involving more specific P-gp inhibitors, are required to definitively elucidate the impact of P-gp inhibition on behavioural responses to antidepressant drugs which are transported substrates of P-gp.

We also investigated the impact of P-gp inhibition on an escitalopram-induced mouse model of serotonin syndrome, finding that CsA pre-treatment significantly exacerbated the manifestation of behaviours associated with this model (*Chapter 5*). This finding highlights the potential for increased risk of side-effects when increasing brain levels of antidepressants by P-gp inhibition. Further studies are required to confirm the mechanism underlying this interaction between CsA and escitalopram, given that CsA was found to modulate escitalopram-induced changes in the serotonergic system as outlined above.

7.7. Do findings from acute experiments in relation to the impact of P-gp inhibition on antidepressant transport across the BBB translate to chronic studies?

One of the main unresolved questions regarding studies demonstrating that P-gp limited the BBB transport of certain antidepressants, and that inhibition of P-gp resulted in increased brain levels of these drugs, was whether these findings would translate to chronic drug administration scenarios which are more analogous to the clinical use of antidepressants. This is a key consideration when contemplating P-gp inhibition as a potential adjunctive strategy for treatment with P-gp substrate antidepressants, given that repeated exposure to xenobiotics, including the antidepressant venlafaxine (Ehret *et al.*, 2007; de Klerk *et al.*, 2010), can result in increased P-gp expression and function (Miller, 2010).

A number of studies had gone some way to addressing this issue by conducting longer term studies in P-gp knockout compared to wild-type mice. However, these

studies could be more aptly described as 'subchronic' rather than chronic *per se*, as they involved drug administration for a maximum of 11 days. Moreover, studies in P-gp knockout mice do not yield information that indicates whether long term administration of a P-gp inhibitor in conjunction with a P-gp substrate antidepressant will result in increased brain levels of the antidepressant. Thus, we conducted a chronic experiment in which the P-gp inhibitor verapamil and the P-gp substrate antidepressant escitalopram were administered daily for 22 days (*Chapter 6*). This study revealed that brain levels of a P-gp substrate antidepressant were indeed increased by P-gp inhibition in a chronic paradigm. This finding indicates that long term treatment with a P-gp inhibitor may represent a viable strategy to achieve enhanced brain delivery of P-gp substrate antidepressants in clinical practice.

In addition, the behavioural and neurochemical effects of antidepressant treatment have been reported to differ between acute and chronic drug administration paradigms (Cryan *et al.*, 2005b; Gaska *et al.*, 2012). Therefore, as a secondary goal of our chronic study, we investigated potential behavioural and molecular consequences of chronically increased brain levels of escitalopram by P-gp inhibition. These experiments did not detect any augmentation of escitalopram's effects by P-gp inhibition. However, there are several potential explanations for this lack of effect, as outlined in Section 6.4, not least the low dose (0.1 mg/kg) of escitalopram administered in the study. Studies which have reported significant effects of chronic antidepressant administration on the mRNA expression of the targets investigated in *Chapter 6* have involved much higher doses than that used in our experiment. The rationale underlying the choice of dose for our chronic study was based on our findings in acute studies, whereby this dose of escitalopram did not elicit a significant antidepressant-like effect when administered on its own, but did when administered in combination with the P-gp inhibitor verapamil. Thus, it seemed rational to investigate the effect of P-gp inhibition on the pharmacodynamic activity of this dose following chronic administration, as using a higher dose of escitalopram would have risked encountering a ceiling effect. Future studies should explore the question of the pharmacodynamic effect of chronic P-gp

inhibitor and antidepressant treatment using a different dosing regimen of escitalopram.

7.8. Limitations of the work outlined in this thesis

No experimental approach is without its limitations, and it is necessary to be cognisant of this fact when interpreting our results. The *in vivo* and *in vitro* techniques used in the work described in this thesis have several limitations, as outlined in the following sections.

7.8.1. Limitations of *in vivo* studies

7.8.1.1. Intracerebral microdialysis

There are several advantages associated with the intracerebral microdialysis technique (see Table 7.1), including the ability to continuously monitor fluctuations in extracellular concentrations of unbound, pharmacologically and physiologically active compounds in specific brain regions, thus offering a unique level of spatial and temporal resolution. However, there are also several important limitations associated with this approach (Table 7.1). For example, stereotaxic implantation of the microdialysis probe causes unavoidable damage to the surrounding tissue, which may in turn affect the integrity of the BBB. The impact of this damage on studies investigating BBB drug transport can be minimised by conducting experiments during the optimal post-surgical period, between 1 and 2 days after probe implantation; after recovery from early tissue reactions and before the start of long term reactions (de Lange *et al.*, 2000). Importantly, in our BBB transport studies, we were able to detect changes in the brain levels of our target compounds as a result of P-gp inhibition, thereby demonstrating that the BBB was exerting a functional effect on drug transport; had the BBB been substantially compromised by the insertion of the microdialysis probe, it is likely that such differences would not have been evident.

Another important limitation associated with our microdialysis studies is that we did not perform quantitative analyses, meaning that we did not determine actual concentrations of the analytes of interest in brain extracellular fluid. Rather, we compared relative changes in analyte concentrations between different treatment

groups. As a result, it was not possible to definitively calculate the actual magnitude of increases in brain concentrations of the antidepressant due to P-gp inhibition. This is a particular concern considering that inhibition of P-gp could alter probe recovery (de Lange *et al.*, 2000). However, both predictions, based on theoretical modelling (Bungay *et al.*, 1990), and experimental evidence (de Lange *et al.*, 1998; Xie *et al.*, 1999) indicate that inhibition of P-gp would result in a reduction in probe recovery of P-gp substrate compounds. Therefore, it is likely that our findings may underestimate the effect of P-gp inhibition on brain levels of antidepressants.

7.8.1.2. Non-selective nature of P-gp inhibitors used

Perhaps the most important limitation of the present work is the non-selective nature of the P-gp inhibitors used, verapamil and CsA. Each of these compounds has an extensive range of pharmacological actions in addition to their inhibition of P-gp. Verapamil is primarily known as a calcium channel blocker, while CsA is a calcineurin inhibitor. Both are clinically used; verapamil for cardiovascular indications, and CsA as an immunosuppressant. The rich pharmacology of these compounds makes isolation of P-gp mediated effects somewhat challenging, especially when investigating pharmacodynamic effects.

The integrated approach adopted in microdialysis-based pharmacokinetic studies allowed us to confirm that increases in brain concentrations following administration of these P-gp inhibitors were not merely a reflection of elevations in circulating blood levels, thereby confirming increased BBB transport. Furthermore, the converging effect of both verapamil and CsA, in enhancing the brain concentrations of imipramine and escitalopram, strongly indicates that this enhancement was mediated via P-gp inhibition, an effect common to both drugs. Moreover, the involvement of P-gp was confirmed by *in vitro* studies, which offered the advantage of elucidating the mechanism at play, in addition to demonstrating that these antidepressants are substrates of human P-gp (as discussed in Section 7.5).

While it was possible to overcome potential problems associated with the non-selective nature of the P-gp inhibitors in pharmacokinetic studies, it is more difficult

to discriminate between P-gp-dependent and P-gp-independent effects in relation to pharmacodynamic studies. We found that verapamil augmented the antidepressant-like activity of escitalopram in the TST, resulting in a reduction in immobility. Moreover, this reduction in immobility was statistically correlated with brain levels of escitalopram, thereby indicating that this effect was likely as a result of increased brain concentrations of escitalopram following P-gp inhibition, as opposed to, for example, verapamil's ability to block calcium channels (Cohen *et al.*, 1997; Busquet *et al.*, 2010). In contrast, inhibition of P-gp by CsA did not augment the antidepressant-like activity of escitalopram in the TST. The effects of CsA on the serotonergic system, as demonstrated by microdialysis studies, and its effect on serotonin turnover in prefrontal cortex brain tissue, in addition to its effects on locomotor activity, indicate that the pharmacodynamic effects of CsA may be acting to suppress any potential enhancement of escitalopram activity due to increased brain levels. Similarly, it is not possible to definitively attribute the exacerbating effects of CsA in an escitalopram-induced mouse model of serotonin syndrome to P-gp dependent or independent mechanisms. Further studies, using more specific P-gp inhibitors (Colabufo *et al.*, 2010), are required to conclusively determine the effect of P-gp inhibition on the pharmacodynamic activity of P-gp substrate antidepressants.

7.8.1.3. Caution extrapolating findings in rodents to humans

As with any *in vivo* preclinical studies, it is important to avoid over-interpretation of our findings in rodents when evaluating their potential relevance to humans. Therefore, the pharmacokinetic differences between rodents and humans need to be highlighted and taken into consideration.

Studies have shown that the capacity of P-gp at the BBB differs between species, requiring different doses of a P-gp inhibitor to elicit the same effect in terms of increased brain distribution of P-gp substrates (Cutler *et al.*, 2006; Syvanen *et al.*, 2009). Thus, it may not be possible to replicate our findings in rodents in human subjects. Indeed, a recent paper, published on behalf of the International Transporter Consortium, has questioned whether clinical modulation of efflux transport at the BBB is likely for these reasons (Kalvass *et al.*, 2013).

Another important consideration, especially relevant to our chronic study, is the difference in drug clearance between rodents and humans. Typically, the half life of a drug is much shorter in rodents than in humans. Owing to their long half lives, most antidepressants accumulate until steady state levels are achieved following chronic once daily administration in humans. In contrast, steady state levels may not be achieved with once, or even more frequent, daily dosing in mice (Frazer and Benmansour, 2002). Thus, it is necessary to be cautious when drawing conclusions from chronic studies involving daily injections in mice. This could be especially relevant when investigating pharmacodynamic effects, as it may be necessary for antidepressant levels to remain consistently above a certain concentration in order to elicit their long term effects (Frazer and Benmansour, 2002). This problem could be overcome with future studies involving continuous drug infusion over a prolonged period of time, for example by using an osmotic minipump.

Similarly, animal models of antidepressant activity, such as the TST used in the present work, have their limitations. The pharmacodynamic findings in these studies do not necessarily mean that similar effects will be observed in patients, as evidenced by the fact that many compounds which have shown antidepressant-like effects in these models have failed to gain clinical approval (O'Leary and Cryan, 2013). Thus, it is necessary to be cautious when interpreting results from our behavioural studies.

7.8.2. Limitations of *in vitro* studies

To ensure accurate identification of antidepressants which are subjected to efflux by P-gp, every effort was made in our *in vitro* work to adopt a rational and consistent methodological approach, based on published guidelines and best practice (Polli *et al.*, 2001; Youdim *et al.*, 2003; Balimane *et al.*, 2006; Zhang *et al.*, 2006). However, it remains a possibility that aspects of our approach may have resulted in misleading results. It is necessary to consider such potential sources of error, as discussed in the following sections.

7.8.2.1. Non-BBB characteristics of cells used for in vitro studies

Due to limitations of BBB-derived systems, as outlined in Section 1.2.5.2, we chose to use cells from a non-cerebral origin for our *in vitro* work. This is not ideal, given that the properties of the endothelium/epithelium are known to influence P-gp activity at the BBB (Romsicki and Sharom, 1999; Hellinger *et al.*, 2012). However, the available data indicate that the MDCKII-MDR1 model we employed is at least as effective, if not more so, at identifying transported P-gp substrates at the BBB than alternative, BBB-derived, *in vitro* approaches (Hellinger *et al.*, 2012). Nonetheless, it is important to bear in mind that our *in vitro* system is not a model of the BBB *per se*, and therefore does not reflect the complexity of uptake, efflux and metabolic processes ongoing at the BBB *in vivo*. Rather, our model represents a tool for the identification of P-gp substrates in general, and this caveat needs to be borne in mind when considering the relevance of *in vitro* findings to BBB transport *in vivo*. Our approach, whereby rodent studies to assess the role of P-gp in the BBB transport of imipramine and escitalopram *in vivo* were combined with *in vitro* studies involving human P-gp, helps to offset this limitation.

7.8.2.2. Concentration-dependence of in vitro studies

The donor concentration used in *in vitro* studies is known to influence the results obtained (Balimane *et al.*, 2006). Using too high a donor concentration may result in saturation of the efflux transporter and a resultant overwhelming of the efflux effect. Conversely, it has been reported that the antiepileptic drug phenobarbital was detected as a P-gp substrate when higher, but not lower, donor concentrations were used (Zhang *et al.*, 2010). Thus, it is possible that the antidepressants which we did not find to be affected by P-gp efflux may have been identified as P-gp substrates had we used a different donor concentration. As a result, further studies are required before these antidepressant drugs can be definitively categorised as non-P-gp substrates.

When attempting to conduct traditional bidirectional transport studies at low donor concentrations, the small amount of the compound of interest that has accumulated in the receiver chamber, in particular at earlier time-points, can present a substantial analytical challenge. The concentration-equilibrium-transport-

assay (CETA) approach may represent a useful means to circumvent this problem in future studies (Luna-Tortos *et al.*, 2008; Zhang *et al.*, 2011). The CETA approach involves simultaneously adding equal concentrations of the compound of interest to both the apical and basolateral chambers. If the compound is a transported P-gp substrate, it will preferentially accumulate in the apical chamber, due to the influence of P-gp efflux at the apical membrane. Thus, due to the addition of the compound to each chamber, the same level of sensitivity in the analytical technique is not required as would be the case for traditional bidirectional transport studies involving the same initial concentration.

7.9. Is P-gp inhibition a potentially viable option to augment antidepressant treatment clinically?

Our work has shown that P-gp inhibition results in increased brain levels of certain antidepressants, and can lead to an augmentation of their antidepressant-like activity, in rodents. This has raised the possibility that adjunctive therapy with a P-gp inhibitor could represent a potential strategy to augment antidepressant treatment in treatment-resistant depression. However, despite recent advances, several outstanding questions remain to be answered before this strategy can be considered a viable clinical option. Some of these questions will be outlined in the following sections.

7.9.1. Which antidepressants are transported substrates of P-gp?

To rationally choose which antidepressant drugs may be amenable to augmentation with a P-gp inhibitor, it is necessary to know which antidepressants are transported P-gp substrates and which are not. In a recent review paper (Zhang *et al.*, 2012), a set of criteria for defining the P-gp substrate status of compounds was proposed in relation to antiepileptic drugs (Table 7.2).

Table 7.2: Definitions of substrate status classifications (Zhang *et al.*, 2012)

Substrate status	Definition
Definite P-gp substrate	Evidence from both *in vivo* (animal model or human data) and *in vitro* bidirectional transport studies demonstrating transport by P-gp
Probable P-gp substrate	Drug is a transported substrate of P-gp *in vitro* but no (or inconclusive) *in vivo* data is available, or vice versa
Possible P-gp substrate	Inconsistent results from *in vitro* and *in vivo* studies
Unlikely to be a P-gp substrate	Drug is not transported by P-gp *in vitro*, but no (or inconclusive) *in vivo* data is available, or vice versa
Not a P-gp substrate	Both *in vivo* and *in vitro* studies have demonstrated that the drug is not transported by P-gp

By adapting these criteria and applying them to antidepressant drugs, it is evident that the P-gp substrate status of most antidepressant drugs remains unclear (Table 7.3). Due to a paucity of data from studies which have investigated if antidepressant drugs are P-gp substrates in humans, we have included pharmacogenetic associations showing alterations in antidepressant pharmacokinetics or treatment response as a surrogate indicator of interactions with human P-gp. Furthermore, we have subdivided animal studies into those using P-gp knockout mice and those involving P-gp inhibition in wild-type animals.

Based on the research outlined in this thesis, in conjunction with recent work carried out by others (Karlsson *et al.*, 2013), escitalopram is the only antidepressant which can be classified as a 'definite' P-gp substrate. In all likelihood, several other antidepressants, including the commonly prescribed citalopram, paroxetine and venlafaxine, are also P-gp substrates, but further studies are required in order to state this with confidence. The lack of clarity in this regard is due, in large part, to deficiencies in relation to *in vitro* studies of antidepressant transport by P-gp. Most of the currently available data from *in vitro* experiments come from only two studies, and there are certain limitations associated with the methodologies employed in these studies (as outlined in Section 7.5). Interestingly, based on the

available data, it seems unlikely that amitriptyline is a transported substrate of P-gp, despite its inclusion as one of four 'P-gp substrate antidepressants' in the landmark pharmacogenetic study demonstrating associations between SNPs in *ABCB1* and treatment response to antidepressants (Uhr *et al.*, 2008). Thus, further research is required in order to definitively determine the P-gp substrate status of commonly used antidepressant drugs before the use of P-gp inhibitors as adjunctive agents can be advocated.

Table 7.3: Antidepressants as P-gp substrates in research models (adapted from studies summarised in *Chapter 1* and the research outlined in this thesis)

AD	Substrate status	*In vitro*[1]	Animals		Humans	Pharmacogenetic associations[2]	
			P-gp knockout	P-gp inhibition		Pharmacokinetic	Response/side effects
Escitalopram	Definite	Y	Y	Y	-	-	Y
Fluvoxamine	Probable	?	Y	-	-	Y	-
Imipramine	Probable	?	-	Y	-	-	-
Protriptyline	Probable	Y	-	-	-	-	-
Citalopram	Possible	N	Y	-	-	Y	?
Desipramine	Possible	N	-	Y	-	-	-
Doxepin	Possible	N	Y[3]	-	-	-	-
Nortriptyline	Possible	N	Y	Y	-	-	?
Paroxetine	Possible	N	Y	-	Y	-	?
Trimipramine	Possible	N	Y	-	-	-	-
Venlafaxine	Possible	N	Y	-	-	-	Y
Amitriptyline	Unlikely	N	?	-	-	-	?
Clomipramine	Unlikely	N	-	-	-	-	-
Duloxetine	Unlikely	N	-	-	-	-	N
Fluoxetine	Unlikely	N	?	-	-	-	N
Trazodone	Unlikely	N	-	-	-	-	-

AD	Substrate status	*In vitro*[1]	Animals		Humans	Pharmacogenetic associations[2]	
			P-gp knockout	P-gp inhibition		Pharmacokinetic	Response/side effects
Mirtazapine	Not	N	N	-	-	-	N
Sertraline	Not	N	N	-	-	-	-
Agomelatine	Unknown	-	-	-	-	-	-
Dosulepin	Unknown	-	-	-	-	-	-
Lofepramine	Unknown	-	-	-	-	-	-
Y = yes; N = no; - = not reported; ? = inconclusive evidence							
[1] Most of the *in vitro* data comes from only two studies which reported almost universally negative data. Due to methodological limitations of these studies, further investigations are needed							
[2] Surrogate indicator of interactions with P-gp in humans							
[3] The increase in P-gp knockout mice relative to WT controls was marginal (10%), but statistically significant							

7.9.2. Would adjunctive treatment with a P-gp inhibitor result in increased brain levels of a P-gp substrate antidepressant in patients?

Due to documented species differences in the ability of P-gp inhibitors to enhance brain distribution of P-gp substrates (Cutler *et al.*, 2006; Syvanen *et al.*, 2009), human studies are needed to demonstrate that P-gp inhibition would result in enhanced brain distribution of antidepressants which are proven to be P-gp substrates. Given that it is generally not possible to directly measure brain levels of a drug in human brain tissue (with the exception being in rare brain biopsy cases), imaging approaches may represent the best approach to assess the impact of P-gp inhibition on the brain distribution of an antidepressant. Ideally, such studies would involve direct measurement of a radiolabelled antidepressant by PET. However, logistical reasons may preclude the generation of such radiolabelled compounds. Measuring serotonin transporter (SERT) occupancy by means of imaging, using selective ligands such as ^{123}I-ADAM, could represent an alternative approach to address this question. Interestingly, this technique has been shown to be sufficiently sensitive to detect a 2-fold difference in the dose of escitalopram treatment, meaning that it may be suitable for this purpose (Klein *et al.*, 2006). Furthermore, the effect of chronic treatment with a P-gp inhibitor and P-gp substrate antidepressant would need to be investigated in man, due to potential species differences as outlined in Section 7.8.1.3.

7.9.3. How significant is the contribution of P-gp efflux to antidepressant resistance?

The causes of treatment resistant depression (TRD) are, in all probability, multifactorial. As such, it is not likely that P-gp inhibition will represent an appropriate augmentation strategy for all refractory depressed patients. However, P-gp mediated restriction of brain levels of antidepressants may contribute to the aetiology of TRD in a subset of patients, especially considering the robust association between P-gp genotype and response to treatment with P-gp substrate antidepressants reported by Uhr and colleagues (Uhr *et al.*, 2008). However, given that the functional effects of SNPs in the *ABCB1* gene are unknown, the exact

mechanism underlying this pharmacogenetic association is unclear. Future imaging studies may serve to elucidate the functional effect of *ABCB1* genotype, thereby helping to inform decisions regarding which patients are likely to benefit from adjunctive P-gp inhibition. Moreover, imaging studies could help to determine if P-gp activity is elevated in patients who fail to respond to antidepressant treatment. Such studies could serve to experimentally evaluate the contribution of P-gp efflux to treatment failure, as current evidence is inconclusive.

7.9.4. Clinical promise and limitations of increasing brain distribution of P-gp substrate antidepressants: Towards an antidepressant augmentation strategy?

We have demonstrated that P-gp inhibition by verapamil enhances the antidepressant-like activity of escitalopram in the tail suspension test, thereby raising the possibility that P-gp inhibition may represent a potential strategy to augment treatment with P-gp substrate antidepressant. However, further preclinical studies are required to more thoroughly investigate the effect of P-gp inhibition on antidepressant-like activity in different animal models and using more specific P-gp inhibitors. Such studies will facilitate informed prediction of expected effects in patients. However, should preclinical investigations yield positive results, it will remain necessary to conduct clinical studies to confirm whether findings from animal models will translate to humans. At present, despite one promising result from our preclinical studies (O'Brien *et al.*, 2013b; *Chapter 4*), there is insufficient data available to conclusively state whether increasing the brain distribution of a P-gp substrate antidepressant by P-gp inhibition would be expected to result in an augmentation of its efficacy as an antidepressant.

Despite the normal viability of P-gp knockout mice (Schinkel *et al.*, 1997) and the tolerability of certain P-gp inhibitors in humans (Kreisl *et al.*, 2010), there are likely to be myriad problems associated with long term clinical use of P-gp inhibitors. P-gp fulfils an important function in several organs in the body, not only at the BBB (Marzolini *et al.*, 2004). Thus, inhibition of P-gp activity could have undesirable effects on the distribution and metabolism of various endogenous and exogenous P-gp substrates. This would be a particular problem in patients taking other

medications which are P-gp substrates, in addition to the antidepressant and P-gp inhibitor. Moreover, although increasing brain levels of an antidepressant drug by P-gp inhibition may facilitate dose reduction while maintaining therapeutic effect, thereby minimising peripheral side-effects, this could prove to be problematic. For example, inceasing brain levels of the antidepressant may lead to an increased risk of centrally-mediated side effects of antidepressant treatment, such as nausea, vomiting and appetite dysregulation, as outlined in Section 1.4.3. Indeed, our research revealed that co-administration of the P-gp inhibitor CsA exacerbated the manifestation of an escitalopram-induced mouse model of serotonin syndrome, thereby highlighting the potential for serious adverse interactions between a P-gp inhibitor and an SSRI (*Chapter 5*). However, additional research is required to determine if this effect was due to P-gp inhibition or an alternative effect of CsA. Regardless, there would be genuine cause for concern about the potential for adverse reactions in patients treated chronically with a P-gp inhibitor. Further studies are needed to evaluate the risk:benefit ratio associated with any putative augmentation of antidepressant treatment by P-gp inhibition.

Furthermore, while the P-gp inhibitors used in our research, verapamil and CsA, have the advantage of being clinically approved and widely available, they lack the requisite potency and specificity to represent viable agents for achieving P-gp inhibition in clinical use (Kalvass *et al.*, 2013). More selective second or third generation P-gp inhibitors, such as the CsA analogue valspodar or tariquidar (which have been used in clinical trials), may represent better alternatives. However, the high cost of more selective P-gp inhibitors such as these may preclude their widespread use in clinical practice. Furthermore, it is unclear whether pharmaceutical companies would be interested in supporting the large scale clinical trials that would be needed in order to investigate the benefit, or lack thereof, of adjunctive P-gp inhibitor treatment in depression, given the previous failures of similar studies in the field of oncology (Szakacs *et al.*, 2006).

7.10. Overall conclusions and future perspectives

In this thesis, we have conducted research which has furthered our understanding of the role played by P-gp in the brain distribution and antidepressant-like activity of antidepressant drugs. To build on this work, further studies are now needed to address unanswered questions regarding the role of P-gp in the pharmacokinetics and pharmacodynamics of antidepressant drugs. For the majority of antidepressants, the available data are inadequate to allow definitive classification as transported substrates of P-gp or not. Further preclinical studies, with a particular focus on *in vitro* approaches involving human P-gp, are required to provide more conclusive information in this regard. Furthermore, additional studies assessing the impact of P-gp inhibition on the effects of antidepressants in animal models of depression are needed to elucidate the pharmacodynamic effect of increasing brain concentrations of P-gp substrate antidepressants. Moreover, clinical studies are required to investigate potential associations between TRD and P-gp efflux of antidepressants. Taken together, the results from such studies will facilitate an informed evaluation of the potential of P-gp inhibition as a novel augmentation strategy in the treatment of depression. Given the potential difficulties associated with therapeutic use of P-gp inhibitors in clinical practice, tailoring the choice or dose of antidepressant based on information relating to P-gp function may represent a more feasible option. Indeed, a pilot clinical study has recently reported that antidepressant treatment can be optimized by the clinical application of *ABCB1* genotyping (Breitenstein *et al.*, 2013). Further investigations of the benefits of this approach will be of interest.

In conclusion, we have demonstrated that the antidepressants imipramine and escitalopram are transported substrates of human P-gp *in vitro* and that acute inhibition of P-gp increases the brain distribution of these antidepressants in rodents. In addition, a further study revealed that findings from acute *in vivo* P-gp inhibition experiments also apply after chronic administration for escitalopram. In contrast, the antidepressants amitriptyline, duloxetine, fluoxetine and mirtazapine were not found to be transported substrates of human P-gp *in vitro*. Moreover, we have shown, for the first time, that inhibition of P-gp can result in an augmentation

of the behavioural effects of an antidepressant. Taken together these findings indicate that P-gp inhibition may represent a potential strategy to augment treatment with certain antidepressants, such as escitalopram. The promise of this approach now requires clinical validation.

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Appendix A: Abbreviations

5-HIAA: 5-hydroxyindoleacetic acid

5-HT: serotonin

5-HTP: 5-hydroxytryptophan

A→B: apical-to-basolateral permeability

ABC: adenosine triphosphate binding-cassette

ACTH: adrenocorticotropic hormone

AF6: afadin

ATP: adenosine triphosphate

AUC: area under the concentration-time curve

B→A: basolateral-to-apical permeability

BBB: blood-brain barrier BCEC: brain capillary endothelial cell

BCEC: brain capillary endothelial cell

BCRP: breast cancer resistance protein

BCSFB: blood-cerebrospinal fluid barrier

C_0: initial concentration following bolus intravenous administration (*Chapter 2*) or initial donor concentration used in *in vitro* studies (*Chapters 3 and 4*)

Caco-2: human colorectal adenocarcinoma cells

Calcein AM: calcein acetoxymethyl ester

CAR: coxsackie- and adenovirus receptor

Cl: drug clearance from plasma

CNS: central nervous system

cTR: corrected (B→A)/(A→B) transport ratio

CRF: corticotrophin-releasing factor

CsA: cyclosporin A

CYP: cytochrome P450

DBS: deep brain stimulation

ECF: extracellular fluid

ECT: electroconvulsive therapy

ESAM: endothelial selective adhesion molecules

FST: forced swim test

HPA axis: hypothalamic-pituitary-adrenal axis

HPLC: high performance liquid chromatography

HPLC-ECD: HPLC with electrochemical detection

IC_{50}: half maximal inhibitory concentration

ICV: intracerebroventricular

JAM: junctional adhesion molecules

JACOP: junction associated coiled-coil protein

k_{el}: elimination rate constant

K_m: Michaelis constant

KO:WT B/P ratio: brain/plasma drug concentration ratios between P-gp knockout and wild-type mice

MAOI: monoamine oxidase inhibitor

MDCK: Madine-Darby canine kidney cells

MDCK-MDR1: *ABCB1*-transfected MDCK cells which overexpress human P-gp

MDCK-WT: wild-type Madine-Darby canine kidney cells

MDR1: multidrug resistance 1 gene

MRP: multidrug resistance-associated proteins

MUPP1: multi-PDZ-protein 1

NaSSA: noradrenergic and specific serotonergic antidepressant

NBD: nucleotide binding domain

NSCLC: non-small cell lung cancer

OB: olfactory bulbectomy

OPA: orthophosphoric acid

P_{app}: apparent permeability coefficient ($cm.s^{-1}$)

PECAM: platelet-endothelial cell adhesion molecule

PFC: prefrontal cortex

P-gp: P-glycoprotein

PK: pharmacokinetic

SBP: substrate-binding pocket

SNP: single nucleotide polymorphism

SNRI: serotonin–noradrenaline reuptake inhibitor

SSRI: selective serotonin reuptake inhibitor

STAR*D: Sequenced Treatment Alternatives to Relieve Depression study

$t_{1/2}$: half-life of drug in plasma

TCA: tricyclic antidepressant

TEER: trans-epithelial electrical resistance

TR: (B→A)/(A→B) transport ratio

TRD: treatment resistant depression

TST: tail suspension test

V_d: volume of distribution

VE-cadherin: vascular endothelial cadherin

VNS: vagus nerve stimulation

ZO: zona occludens proteins

Appendix B: Publications and presentations

B.1. Peer-reviewed publications

O'Brien FE, O'Connor RM, Clarke G, Donovan MD, Dinan TG, Griffin BT and Cryan JF. The P-glycoprotein Inhibitor Cyclosporin A Differentially Influences Behavioural and Neurochemical Responses to the Antidepressant Escitalopram. *Behavioural Brain Research* doi: 10.1016/j.bbr.2013.11.027. [Epub ahead of print]

O'Leary OF, O'Brien FE, O'Connor RM and Cryan JF (under review). Drugs, Genes and the Blues: Pharmacogenetics of the Antidepressant Response from Mouse to Man. *Pharmacology Biochemistry and Behavior* doi: 10.1016/j.pbb.2013.10.015. [Epub ahead of print]

O'Brien FE, Clarke G, Dinan TG, Cryan JF and Griffin BT (2013). Human P-glycoprotein Differentially Affects Antidepressant Drug Transport: Relevance to Blood-Brain Barrier Permeability. *The International Journal of Neuropsychopharmacology 16(10):2259-2272*

O'Brien FE, O'Connor RM, Clarke G, Dinan TG, Griffin BT and Cryan JF (2013). P-glycoprotein Inhibition Increases the Brain Distribution and Antidepressant-Like Activity of Escitalopram in Rodents. *Neuropsychopharmacology 38(11):2209-19*

Browne CA, O'Brien FE, Connor TJ, Dinan TG and Cryan JF (2012). Differential lipopolysaccharide-induced immune alterations in the hippocampus of two mouse strains: effects of stress. *Neuroscience 225:237-48*

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B.2. Manuscripts in preparation:

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B.3. Conference presentations

F.E. O'Brien, R.M. O'Connor, G. Clarke, M.D. Donovan, T.G. Dinan, B.T. Griffin, J.F. Cryan (2013). An investigation of pharmacokinetic and pharmacodynamic interactions between the P-glycoprotein inhibitor Cyclosporin A and the antidepressant escitalopram. *Neuroscience Ireland*, 12-13 September 2013, Cork, Ireland.

FE O'Brien, O'Connor RM, Clarke G, Dinan TG, Griffin BT, Cryan JF (2013). Effect of P-glycoprotein inhibition on the brain distribution and antidepressant-like activity of escitalopram in rodents (oral). *Royal Academy of Medicine in Ireland Section of Biomedical Sciences Annual Meeting*, 20 June 2013, Cork, Ireland.

FE O'Brien, Clarke G, Dinan TG, Griffin BT, Cryan JF (2013). P-glycoprotein restricts blood-brain barrier transport of the antidepressant escitalopram: converging evidence from *in vitro* and *in vivo* studies (poster). *Beating the Blood-Brain and other Blood Barriers Conference*, 6-8 February 2013, Lisbon, Portugal.

FE O'Brien, Clarke G, Dinan TG, Griffin BT, Cryan JF (2012). The Influence of P-glycoprotein on the Blood-Brain Barrier Transport of the Antidepressant Escitalopram: Converging Evidence from *In vivo* and *In vitro* Studies (oral). *British Pharmacological Society Winter Meeting*, 18-20 December 2012, London, UK.

FE O'Brien, Clarke G, Dinan TG, Cryan JF, Griffin BT (2012). P-glycoprotein limits antidepressant transport across the blood-brain barrier: converging evidence from *in vitro* and *in vivo* studies (poster). *American Association of Pharmaceutical Scientists Annual Meeting and Exposition*, 14-17 October 2012, Chicago, IL, USA.

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FE O'Brien, Clarke G, Fitzgerald P, Dinan TG, Griffin BT, Cryan JF (2012). P-glycoprotein inhibition enhances the transport of the antidepressant imipramine across the blood-brain barrier (poster). *European College of Neuropsychopharmacology Workshop on Neuropsychopharmacology for Young Scientists in Europe*, 15-18 March 2012, Nice, France.

FE O'Brien, Clarke G, Fitzgerald P, Dinan TG, Griffin BT, Cryan JF (2012). Inhibition of P-glycoprotein Enhances Transport of the Antidepressant Imipramine across the Blood-Brain Barrier: Microdialysis Studies in the Conscious Freely Moving Rat (oral). *All-Ireland Joint Schools of Pharmacy Research Conference*, 3 April 2012, Cork, Ireland.

FE O'Brien, Clarke G, Fitzgerald P, Dinan TG, Griffin BT, Cryan JF (2011). The Influence of P-gp Inhibition on Imipramine Transport across the Blood-Brain Barrier: Microdialysis Studies in the Conscious Freely Moving Rat (poster). *British Pharmacological Society Winter Meeting*, 13-15 December 2011, London, UK.

FE O'Brien, Clarke G, Fitzgerald P, O'Driscoll CM, Dinan TG, Cryan JF, Griffin BT (2011). Evaluation of the influence of P-glycoprotein inhibition on plasma and brain pharmacokinetics of the antidepressant imipramine: microdialysis studies in the conscious freely moving rat (poster). *American Association of Pharmaceutical Scientists Annual Meeting and Exposition*, 23-27 October 2011. Washington DC, USA.

FE O'Brien, Clarke G, Fitzgerald P, Dinan TG, Griffin BT, Cryan JF (2011). Evaluation of the influence of P-glycoprotein inhibition on plasma and brain pharmacokinetics of the antidepressant imipramine: microdialysis studies in the conscious freely moving rat (poster). *Neuroscience Ireland*, 1-2 September 2011, Kildare, Ireland.

FE O'Brien, Clarke G, Fitzgerald P, Griffin BT, Cryan JF (2011). An integrated microdialysis model for the analysis of blood-brain barrier drug transport in the conscious freely moving rat (poster). *All-Ireland Joint Schools of Pharmacy Research Conference*, 18-19 April 2011, Dublin, Ireland.

Appendix C:
Establishment of surgical and analytical techniques

C.1. Introduction

In order to evaluate the BBB transport of a compound using microdialysis, it was necessary to develop an integrated technique which enables the simultaneous determination of drug levels in the brain and the blood (Bundgaard *et al.*, 2007). This integrated technique involves concomitant intracerebral microdialysis and peripheral catheterizations. To facilitate intravenous drug administration (thereby avoiding variability in drug absorption) and serial blood sampling (to determine plasma drug pharmacokinetics), in-dwelling catheters are surgically inserted into the jugular vein and carotid artery respectively, while a microdialysis probe is inserted into the brain to monitor fluctuations in drug concentrations in the brain extracellular fluid (ECF).

It was decided that achieving proficiency at each of these intricate surgical procedures individually was necessary before attempting to combine them in the same animal. To this end, imipramine, a tricyclic antidepressant drug, was selected as the model drug during method development. Imipramine was chosen as it has previously been sampled using the microdialysis technique (Sato *et al.*, 1994), and the ability of antidepressants such as imipramine to cross the BBB has come under scrutiny of late (O'Brien *et al.*, 2012b). It was therefore also necessary to develop a highly sensitive analytical assay to facilitate detection of the very low levels of imipramine that were anticipated to be recovered in the microdialysis samples. In addition, imipramine has an active metabolite, desipramine, which we aimed to detect and quantify using the same analytical technique.

The present study had several goals. Firstly, it was aimed to develop and optimise a highly sensitive analytical assay which would enable detection and quantification of imipramine down to the nanograms per millilitre concentration range. High performance liquid chromatography with electrochemical detection (HPLC-ECD) was chosen as an appropriate analytical technique for this purpose based on previous reports in the literature (Sato *et al.*, 1994). It was necessary to determine the optimal electrochemical detector settings for the determination of concentrations of imipramine, desipramine and the internal standard trimipramine.

Using these optimised settings, calibration curves for these drugs at low and high concentration ranges, as would be expected in microdialysis and plasma samples respectively, were generated to determine if a linear relationship exists between analyte concentration and detector response. Secondly, once the HPLC method had been established, it was aimed to validate the success of peripheral catheterization surgeries by administering imipramine intravenously followed by serial blood sampling. Using this method, we aimed to generate imipramine and desipramine plasma pharmacokinetic profiles in dual-catheterized rats following intravenous administration and determine an optimal intravenous dose for use in further studies. In order to do this, it was necessary to optimise a plasma extraction technique and to validate that imipramine, desipramine and trimipramine could be reliably and efficiently extracted from plasma samples, and that these compounds in the extracted plasma samples could be determined using the HPLC-ECD system. Initial catheterization surgeries were carried out on a non-recovery basis. Therefore the plasma pharmacokinetics of imipramine were initially determined in anaesthetized animals. Once greater surgical proficiency was achieved, imipramine plasma pharmacokinetics were determined in conscious freely moving animals which had recovered post-operatively. Thirdly, we measured plasma corticosterone concentrations over time in one conscious freely moving catheterized animal following post-operative recovery to ascertain that it was possible to monitor diurnal variations in plasma corticosterone levels using our technique and to determine if plasma corticosterone levels were elevated above expected normal values on the day following surgery. Finally, we aimed to validate the success of our microdialysis technique by detecting fluctuations in imipramine levels in microdialysis samples in the conscious, freely moving rat following intraperitoneal imipramine administration in animals which had received microdialysis surgery only.

C.2. Methods

C.2.1. Drugs and chemicals

High performance liquid chromatography (HPLC) grade acetonitrile, potassium dihydrogen phosphate and orthophosphoric acid (OPA) were obtained from Fisher Scientific (Ireland). Heparin sodium solution (Wockhardt UK Ltd, UK) was purchased from Uniphar Group (Ireland). Imipramine, desipramine, trimipramine and verapamil were obtained from Sigma-Aldrich as were all other chemicals unless otherwise stated.

C.2.2. HPLC method development

C.2.2.1. HPLC equipment

The HPLC with electrochemical detection (HPLC-ECD) system consisted of a Shimadzu LC-20AD XR Prominence Pump, CBM-20A communication bus module, SIL-20AC XR Prominence Autosampler, CTO-20A Prominence Column oven (all supplied by Mason Technology, Cork, Ireland). System components were used in conjunction with Shimadzu LC solutions software (Mason Technology). The detector used was ESA Coulochem III with the 5041 Amperometric Cell (Supplied by ESA Analytical, Ltd., Brook Farm, Dorton, Aylesbury, Buckinghamshire, HP18 9NH England). All samples were injected onto a reversed phase Luna 3 µm C18(2) 150 x 2 mm column (Phenomenex), which was protected by Krudkatcher Ultra in-line filters (Phenomenex).

C.2.2.2. HPLC optimisation and validation

Standard solutions of imipramine, desipramine, verapamil and trimipramine were injected onto the HPLC system at different voltages to determine the optimal voltage. Response (as measured by peak height) was plotted against applied voltage to facilitate identification of the optimal voltage. Calibration curves were generated to investigate if a linear relationship exists between analyte concentration and peak height across the concentration ranges to be investigated.

C.2.2.3. HPLC conditions

The HPLC-ECD method was adapted from previously described methods (Sato *et al.*, 1994; Frahnert *et al.*, 2003). Briefly, the mobile phase which was used on the HPLC

system consisted of a mixture of 25mM potassium dihydrogen phosphate (25 mM, pH 7 with 4N NaOH) and HPLC grade acetonitrile (56:44). Mobile phase was filtered through Millipore 0.22 µm Durapore filters (Millipore, Ireland) and vacuum degassed prior to use. Compounds were eluted isocratically over a 30 min runtime at a flow rate of 0.4 ml.min^{-1} after a 20 µl injection. The column was maintained at a temperature of 30^{o}C and samples/standards were kept at 8^{o}C in the cooled autoinjector prior to analysis. The glassy carbon working electrode combined with a platinum reference electrode (ESA) was operated at a potential of 600mV and a range of 50nA.

C.2.2.4. Analyte identification and quantification

Imipramine, desipramine and trimipramine (internal standard) were identified by their characteristic retention times as determined by standard injections which were run at regular intervals during sample analysis. For the extracted plasma samples, analyte:internal standard peak height ratios were measured and compared with standard injections, and results were expressed as nanograms of analyte per ml of plasma. As no extraction procedure was necessary for microdialysis samples, analyte peak height ratios were compared directly with standard injections and expressed as nanograms of analyte per ml of dialysate

C.2.3. *In vivo* experiments

Male and female Sprague Dawley rats (Harlan Laboratories, UK) were used for these preliminary *in vivo* experiments. Animals were group-housed 4-6 animals per cage and maintained on a 12 hour light/dark cycle (lights on at 08:00 h) with food and water ad libitum. Room temperature was controlled at 22 ± 1°C. All procedures were carried out in accordance with EU directive 89/609/EEC and approved by the Animal Experimentation & Ethics Committee of University College Cork.

C.2.3.1. Development of surgical procedures

Animals were anaesthetized prior to surgery with a ketamine/xylazine mixture (90/10 mg.kg^{-1} i.p.), with maintenance of anaesthesia achieved by repeating 20-25% of the induction dose at 30-40 minute intervals, as required. Analgesia was provided by pre-operative administration of carprofen (3 mg.kg^{-1} s.c.). Throughout

surgical procedures, the body temperature of each rat was maintained using a heating pad.

C.2.3.2. Catheterization surgeries

Two indwelling catheters were surgically implanted in each rat using standard surgical techniques: one in the carotid artery for collection of serial blood samples and one in the external right jugular vein to facilitate intravenous drug administration (Thrivikraman *et al.*, 2002; Huang *et al.*, 2006; Heiser, 2007). All catheters were pyrogen-free polyethylene tubing (Instech Laboratories, Plymouth Meeting, PA). The arterial and venous catheters consisted of BPE-T25 tubing (0.018"ID x 0.036"OD) and BPE-T50 tubing (0.023"ID x 0.038"OD), respectively. The catheters were subcutaneously tunnelled to the back of the neck of the rat, where they were exteriorized and connected to three-way Discofix® stopcocks (B. Braun). To prevent clotting the catheters were filled with a heparinised saline solution (20 $IU.ml^{-1}$).

C.2.3.3. Blood sampling experiments

After successful catheterization surgeries, rats were administered imipramine either intravenously or by oral administration. Initial experiments were carried out on anaesthetized animals, while later experiments were carried out on conscious freely moving animals. In one case where the intravenous line was not patent, imipramine was administered by i.p. injection rather than by i.v. injection. Following imipramine administration, blood samples were taken at regular intervals up to 300 minutes post-imipramine administration. Blood samples (~ 250 µl) were immediately centrifuged at 5000 rpm for 5 minutes, plasma taken and stored at -20^{o}C or -80^{o}C until extraction for analysis by HPLC with electrochemical detection. At the conclusion of the experiment, rats were euthanized by administration of intravenous sodium pentobarbitone (euthatal).

C.2.3.4. Plasma extraction

Imipramine and its active metabolite desipramine were extracted from plasma using a liquid-liquid extraction technique as described previously (Clarke *et al.*, 2009), with some modifications. Briefly, 98 µl of plasma was spiked with 2 µl of the

internal standard, trimipramine, to yield a final concentration of 20 $ng.ml^{-1}$ trimipramine. To this trimipramine-spiked plasma, 1 ml of sodium hydroxide (2 M) and 3 ml of water were added. Extraction was carried out in 7.5 ml of 1.5% isoamyl alcohol in n-heptane by vortexing for 30 seconds, followed by agitation on a mechanical shaker for 15 min and then centrifugation at 5000 RPM for 15 min at 20°C. The upper solvent layer was transferred to a tube containing 200 µl of 25 mM OPA, vortexed for 30 seconds, then agitated on a mechanical shaker for 15 min followed by centrifugation at 5000 rpm for 15 min at room temperature. Twenty microlitres of the lower aqueous phase was injected onto the HPLC system for analysis. This reliability and efficiency of this extraction technique was validated before use by extracting blank plasma samples spiked with known concentrations (100 $ng.ml^{-1}$) of imipramine and desipramine.

C.2.3.5. Determination of diurnal variations in plasma corticosterone concentrations

Serial blood samples (~200 µl) were taken at two-hourly intervals from one drug-naive rat on the day following successful catheterizations. Blood samples were immediately centrifuged at 5000 rpm for 5 minutes at 4°C, plasma taken and stored at -20^{o}C until assayed. Plasma corticosterone concentrations were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, USA) according to the manufacturer's instructions.

C.2.3.6. Development and validation of microdialysis technique

C.2.3.7. Dialysis probe construction and calibration

Vertical concentric microdialysis probes were constructed as described elsewhere (Page and Lucki, 2002). Briefly, a piece of fused silica (ID 75 ± 3 µm, OD 150 ± 6 µm; CM Scientific Ltd, UK) was inserted through PE10 tubing (Instech Laboratories, Plymouth Meeting, PA). A Spectra/Por® Micro-dialysis Hollow Fiber regenerated cellulose semipermeable membrane with a 13 kD molecular weight cut-off (Spectrum Laboratories, Inc.) was placed over the fused silica and into the PE10 tubing, and fixed in place using epoxy adhesive. The open end of the semipermeable membrane was sealed with a 0.5 mm epoxy plug, and the active

area of the fibre, where diffusion takes place across the membrane, was limited to 3 mm in length by coating regions outside this range with epoxy adhesive. *In vitro* recovery experiments were carried out to demonstrate that imipramine and desipramine could be recovered using the microdialysis probes. These *in vitro* recovery experiments involved immersing each probe in a well of aCSF containing a known concentration (100 $ng.ml^{-1}$) of imipramine and desipramine. The concentration of imipramine and desipramine present in the dialysis samples was expressed as a percentage of the known concentration in the well and this percentage is termed the recovery rate.

C.2.3.8. Microdialysis surgery

Rats were placed in a stereotaxic frame (Model 900 Small Animal Stereotaxic Instrument, David Kopf Instruments, Bilaney Consultants, St Julians, Sevenoaks, UK) with the skull flat. A small burr hole was made in the skull, centred 2.7 mm anterior and 0.7 mm lateral to bregma. The microdialysis probe was slowly lowered 5 mm from dura into the prefrontal cortex (PFC) (Paxinos and Watson, 1998) and secured with skull screws and dental acrylic. The inlet of the probe was connected to a fluid swivel (Instech Laboratories, Plymouth Meeting, PA) and the rats were single-housed in cylindrical plexiglass containers (Instech Laboratories, Plymouth Meeting, PA) filled with bedding and food pellets. Artificial cerebrospinal fluid (aCSF: 147 mM NaCl, 1.7 mM $CaCl_2$, 0.9 mM $MgCl_2$, and 4mM KCl) was continuously perfused through each microdialysis probe at a rate of 1.5 $\mu l.min^{-1}$ by a microlitre 'Pico Plus' syringe pump (Harvard Apparatus, Fircroft Way, Edenbridge, Kent UK).

C.2.3.9. Microdialysis sampling experiments

After successful microdialysis surgeries, rats were allowed to recover overnight prior to microdialysis sampling experiments on the following day during the optimal post-surgical period (de Lange *et al.*, 2000). Microdialysis samples were taken before imipramine administration (blanks) and at 20 minute intervals following the administration of imipramine (30 $mg.kg^{-1}$ i.p.) for 300 minutes. Imipramine and desipramine concentrations in the microdialysis samples were determined by HPLC analysis on the same day as they were collected without any extraction procedure.

C.3. Results

C.3.1. Optimal potential for ECD detector

The response (peak height) increased with increasing voltage applied across the electrode at all applied voltages tested for trimipramine and verapamil, while the response increased to a maximum at 700 mV for imipramine before decreasing at a higher voltage (Figure C.1). The response for desipramine reached a plateau at an applied voltage of 600 mV and declined at higher applied voltages. As the background noise increases with increasing applied voltage, it was decided that 600 mV was the optimal voltage at which to analyse our samples.

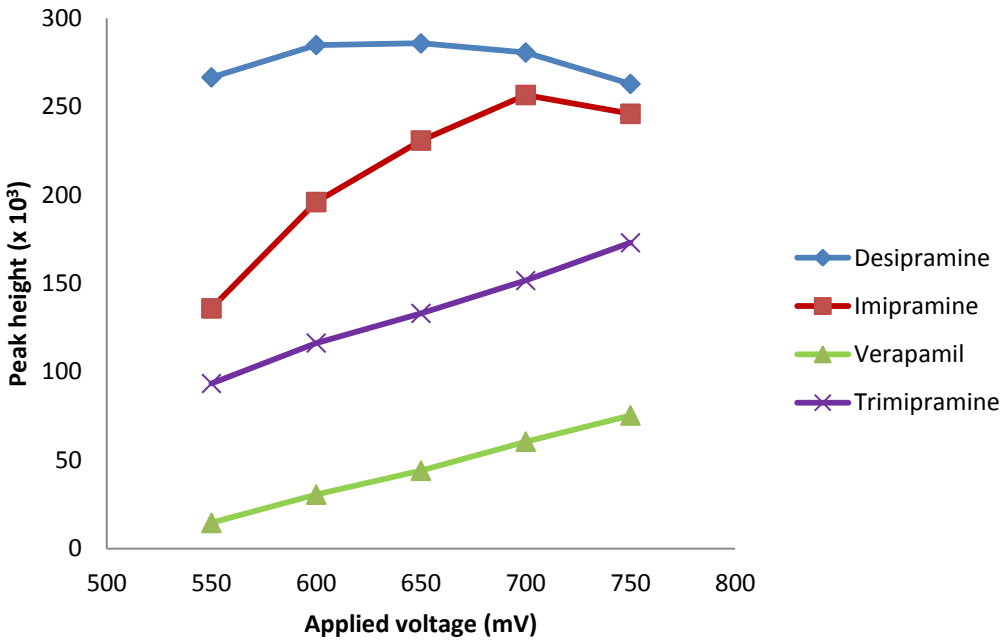

Figure C.1: Determination of optimal potential for ECD detection of analytes

C.3.2. Calibration curves of HPLC method

There was a linear relationship between peak height and concentration for all three drugs tested as demonstrated by the r^2 values listed below, all of which are greater than 0.99 (Figure C.2). This was the case across the entire concentration range (A), at low concentrations (as expected for microdialysis samples: B) and at higher concentrations (as expected in plasma samples: C).

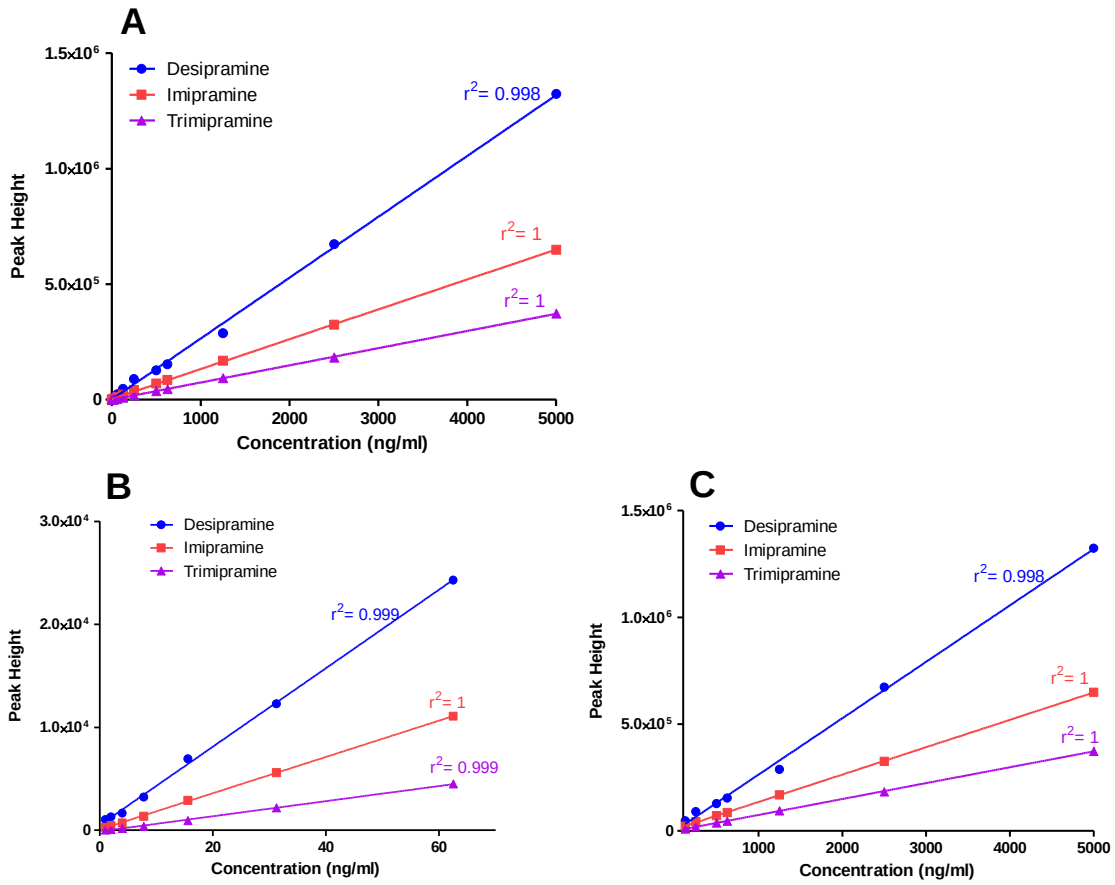

Figure C.2: Calibration curves for imipramine, desipramine and trimipramine on HPLC-ECD

A. Overall calibration curve for imipramine, desipramine and trimipramine over concentration range 1 ng.ml^{-1} to 5000 ng.ml^{-1} with r^2 values listed on graph. B. Calibration curve for imipramine, desipramine and trimipramine at lower concentration range (1 ng.ml^{-1} to 62.5 ng.ml^{-1}) with r^2 values listed on graph. C. Calibration curve for imipramine, desipramine and trimipramine at higher concentration range (125 ng.ml^{-1} to 5000 ng.ml^{-1}) with r^2 values listed on graph.

C.3.3. Plasma extraction efficiency

The extraction efficiency of our liquid-liquid extraction technique was determined by spiking blank plasma with a known concentration of imipramine, desipramine and trimipramine (100 ng.ml^{-1}). The concentration of these drugs in our extracted samples was expressed as a percentage of the initial concentration and termed 'extraction efficiency'. As illustrated in Figure C**.3**, the extraction efficiency for all three compounds was > 80% and the extraction procedure was reliable and reproducible.

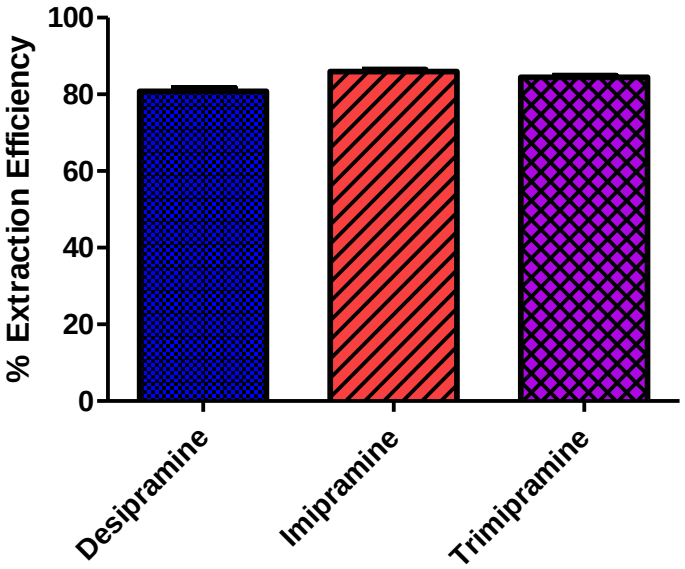

Figure C.3: Plasma extraction efficiency

Percentage of the spiked concentration of desipramine, imipramine and the internal standard trimipramine obtained in extracted samples (n = 4 extractions)

C.3.4. Imipramine and desipramine plasma profiles in anaesthetized rats

C.3.4.1. Following intravenous imipramine (15 mg.kg^{-1}) administration

Imipramine (15 mg.kg^{-1} i.v.) was administered to an anaesthetized rat immediately after successful completion of dual catheterization surgery. Anaesthesia was maintained for 90 minutes post-imipramine administration, and blood samples were taken at regular intervals. The concentration of imipramine and desipramine were determined at each time-point and are presented in the plasma profiles shown in Figure C**.4**.

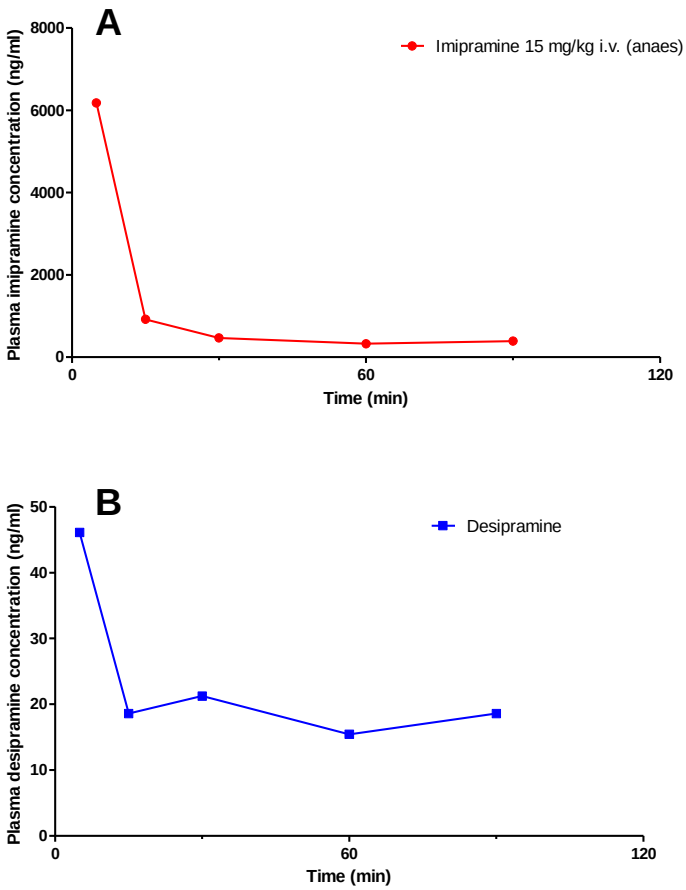

Figure C.4: Imipramine and desipramine plasma profiles in an anaesthetized animal after the intravenous administration of imipramine 15 mg.kg^{-1} (n = 1)

C.3.4.2. Following intraperitoneal imipramine (15 mg.kg^{-1}) administration

Following completion of the dual-catheterization surgery in one anaesthetized animal, the jugular vein catheter was not patent. Therefore, imipramine (15 mg.kg^{-1}) was administered i.p. rather than i.v., and plasma samples were taken at regular intervals up to 90 minutes. Anaesthesia was maintained throughout the sampling procedure. The resulting plasma imipramine and desipramine profiles are presented in Figure C**.5**.

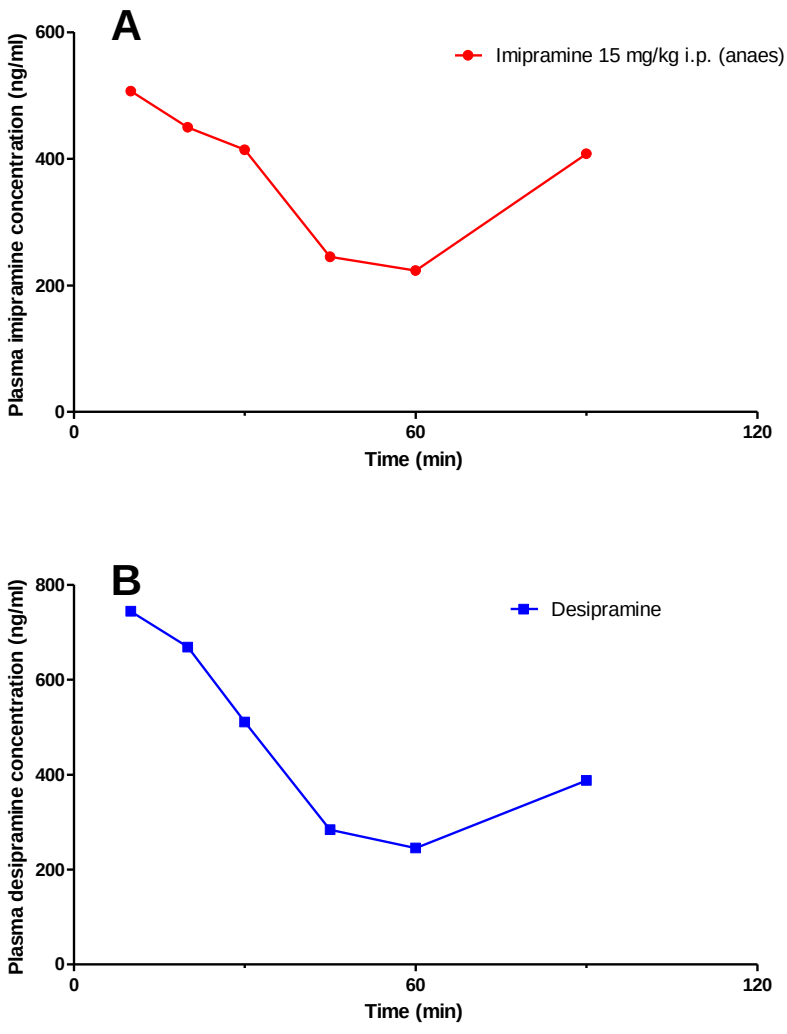

Figure C.5: Imipramine and desipramine plasma profiles in an anaesthetized animal after the intraperitoneal administration of imipramine 15 mg.kg^{-1} (n = 1)

C.3.5. Imipramine and desipramine plasma profiles in conscious freely moving rats

C.3.5.1. Following intravenous imipramine (1 mg.kg^{-1}) administration

Following successful dual-catheterization surgery, one animal was allowed to recover overnight prior to imipramine (1 mg.kg^{-1} i.v.) administration while conscious and freely moving. This dose was tolerated without any adverse reaction, and plasma samples were taken at regular intervals up to 300 minutes post-imipramine administration. The resulting imipramine plasma profile is illustrated in Figure C**.6**. For technical reasons, desipramine concentrations could not be determined in these plasma samples.

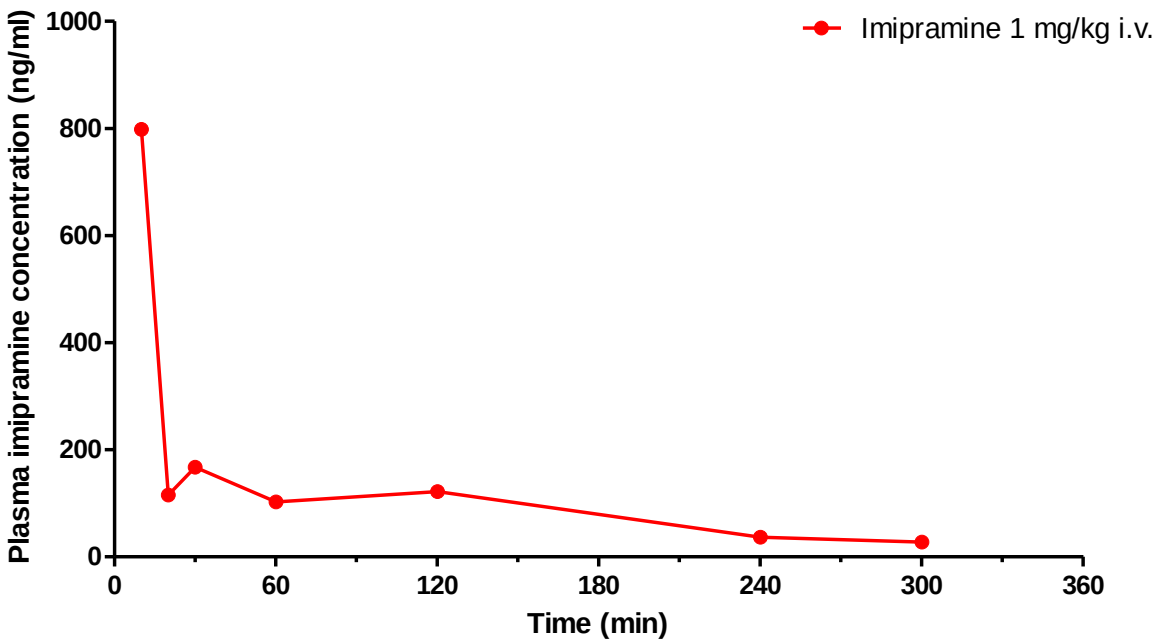

Figure C.6: Imipramine plasma profile in a conscious freely moving animal following the intravenous administration of imipramine 1 mg.kg^{-1} (desipramine levels could not be determined for technical reasons) (n = 1)

C.3.5.2. Following intravenous imipramine (5 mg.kg^{-1}) administration

Following successful dual-catheterization surgery, imipramine (5 mg.kg^{-1} i.v.) was administered to six conscious freely moving animals. This dose was tolerated without any adverse reactions, and plasma samples were taken at regular intervals up to 300 minutes post-imipramine administration. The resulting mean imipramine and desipramine plasma profiles are illustrated in Figure C**.7**.

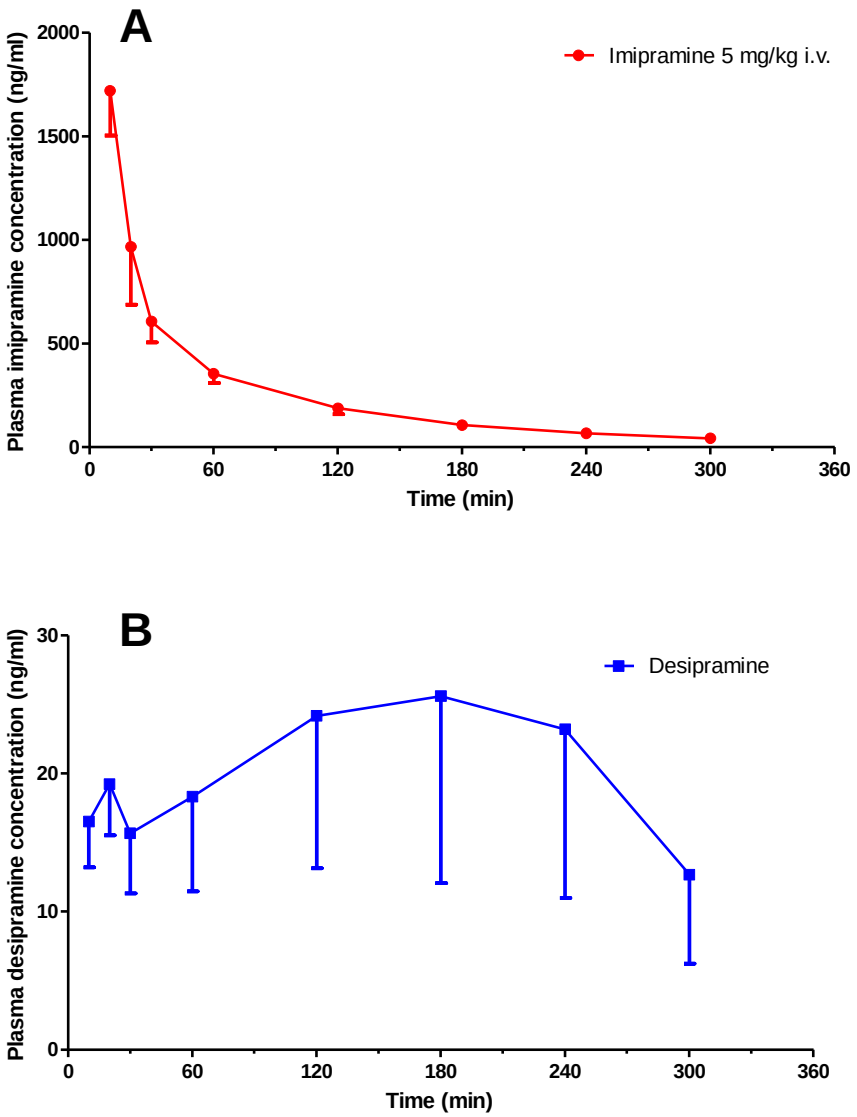

Figure C.7: Imipramine and desipramine plasma profiles in conscious freely moving animals after the intravenous administration of imipramine 5 mg.kg^{-1} (n = 6)

C.3.5.3. Following oral imipramine (30 mg.kg^{-1}) administration

Following successful dual-catheterization surgery, imipramine (30 mg.kg^{-1}) was administered orally to three conscious freely moving animals. Plasma samples were taken at regular intervals up to 300 minutes post-imipramine administration. The resulting mean imipramine and desipramine plasma profiles are illustrated in Figure C.**8**.

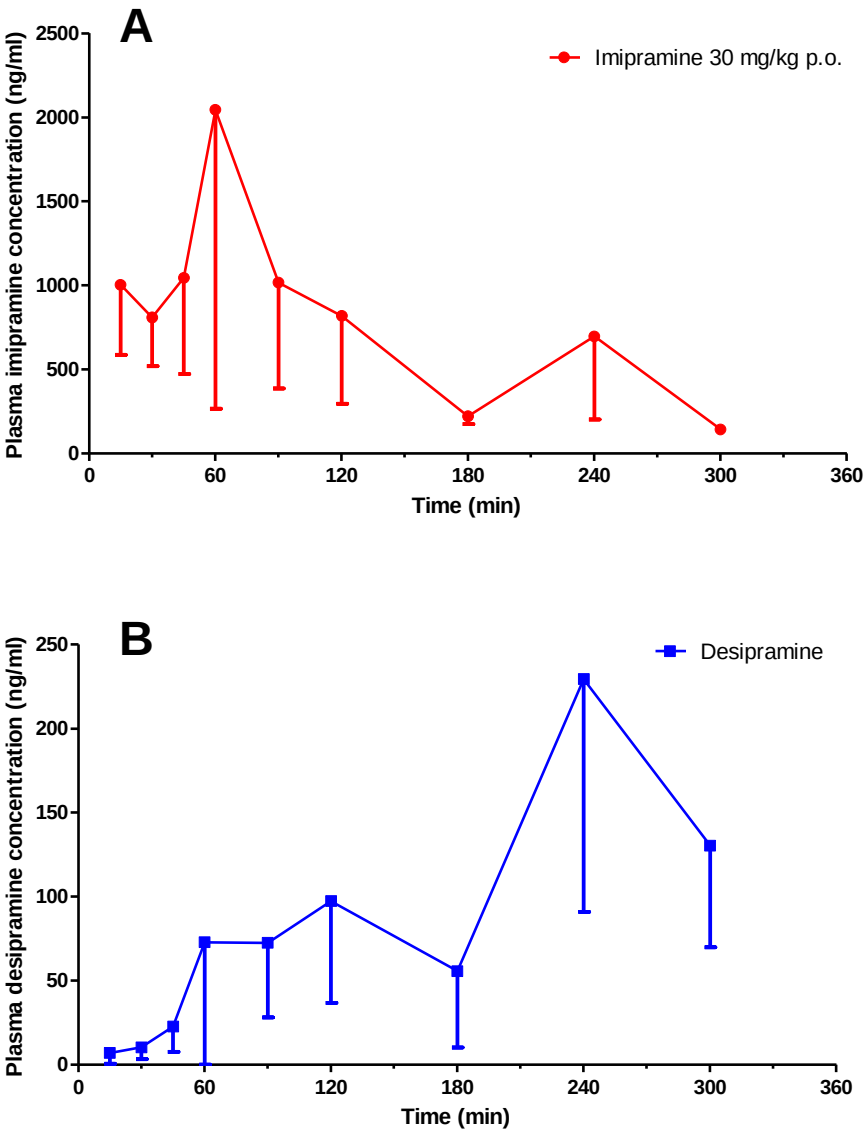

Figure C.8: Imipramine and desipramine plasma profiles in conscious freely moving animals after the oral administration of imipramine 30 mg.kg^{-1} (n = 6)

C.3.6. Diurnal variation in plasma corticosterone levels in a conscious freely moving rat one day after catheterization surgery

Following overnight recovery after successful dual catheterization surgery, plasma samples were taken from one female rat every two hours between 8 am and 10 pm and plasma corticosterone levels were determined in each plasma sample. The resulting plot illustrating the diurnal variation in plasma corticosterone concentrations throughout the day is shown in Figure C**.9**. The overall mean plasma corticosterone concentration was 145.8 ng.ml^{-1}.

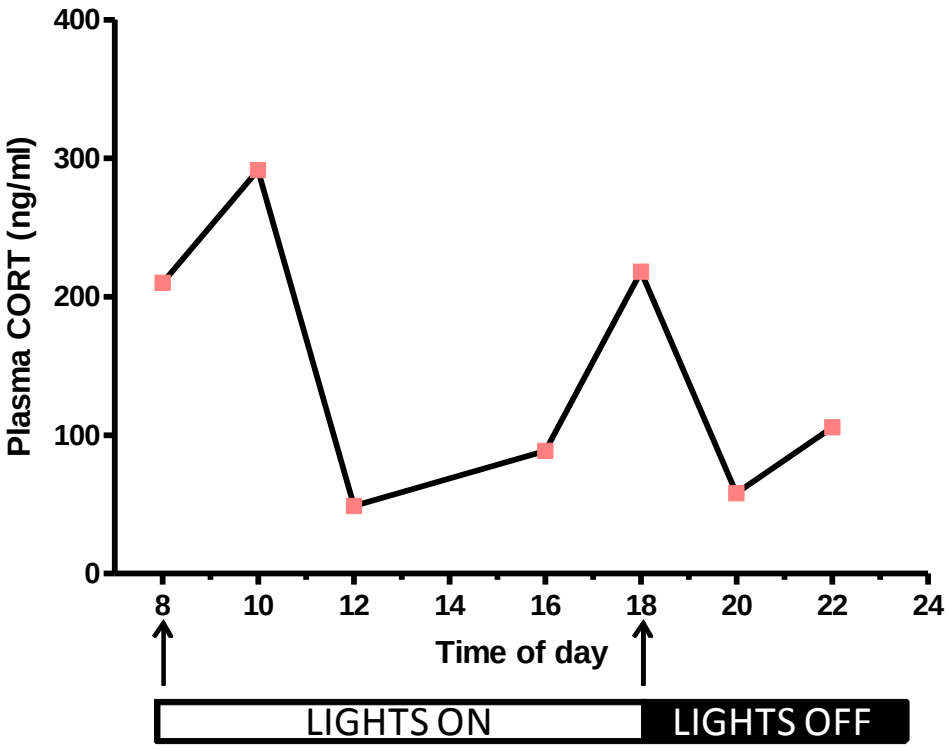

Figure C.9: Diurnal variation in plasma corticosterone levels as measured by serial blood sampling from an indwelling arterial catheter (n=1)

C.3.7. Microdialysis probe recovery – *in vitro* experiments

The mean *in vitro* probe recovery by gain for desipramine (DMI), imipramine (IMI), verapamil (VERAP) and trimipramine (TRIM) are illustrated in Figure C**.10** (n = 6 probes).

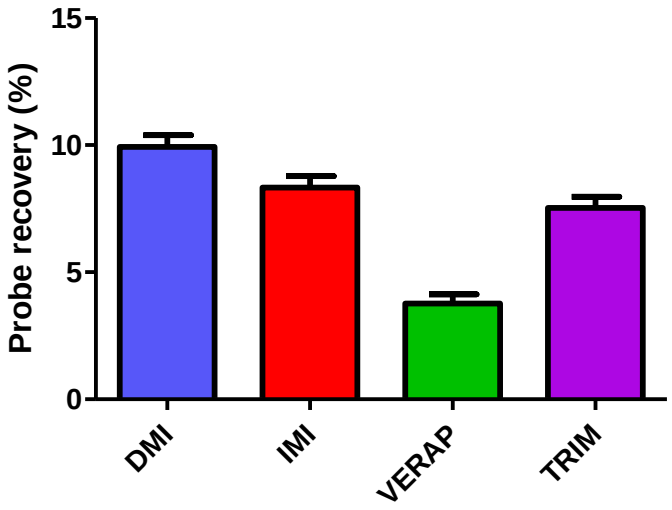

Figure C.10: *In vitro* probe recovery by gain from a 100 ng.ml^{-1} solution (n = 6 probes)

C.3.8. Imipramine and desipramine dialysate profiles in conscious freely moving rats after intraperitoneal imipramine (30 mg.kg^{-1}) administration

Imipramine (30 mg.kg^{-1} i.p.) was administered to 3 rats on the day following successful microdialysis surgery and the resulting concentration of imipramine and desipramine in the microdialysis samples at different time intervals post-imipramine administration was measured using HPLC-ECD. The imipramine and desipramine levels in all samples were quantifiable, and the resulting profiles are shown in Figure C**.11**.

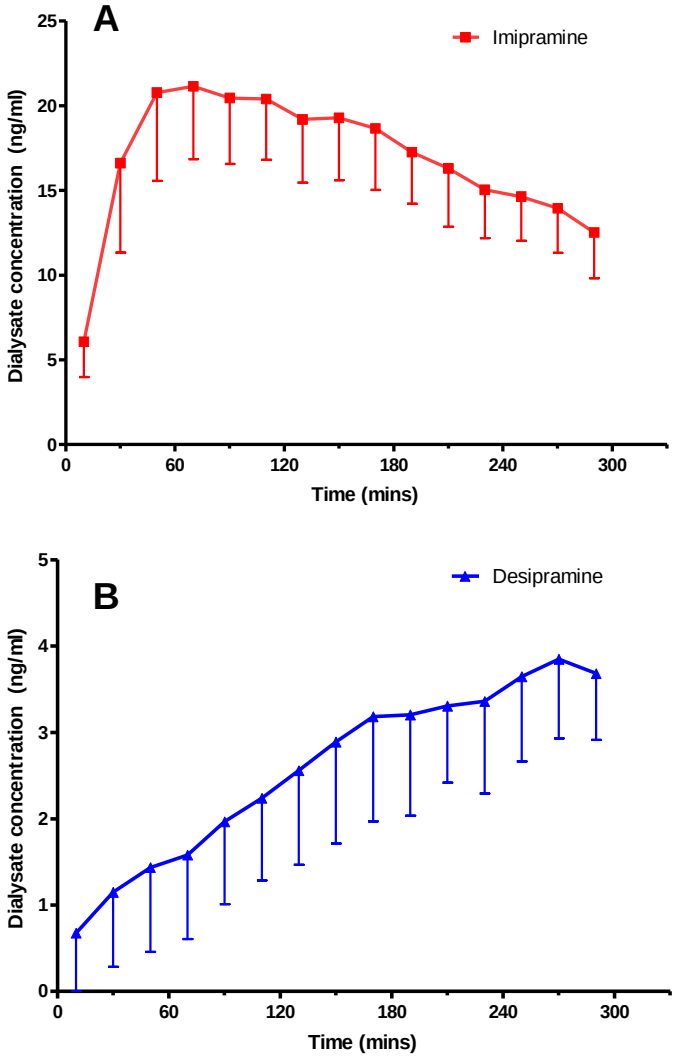

Figure C.11: Dialysate imipramine and desipramine concentration-time profiles following intraperitoneal imipramine administration (30 mg.kg^{-1}) (n=3)

C.4. Discussion

The present data demonstrate the success of our analytical and surgical techniques. We successfully developed a highly sensitive analytical technique which facilitated the determination of imipramine, desipramine and the internal standard trimipramine concentrations in the $ng.ml^{-1}$ range. Furthermore, we successfully developed a surgical catheterization technique which facilitated the intravenous administration of imipramine via the jugular vein and serial blood sampling via the carotid artery. Using this catheterization technique, it was possible to repeatedly remove small volumes of blood (plasma) from a conscious freely moving animal after intravenous imipramine administration. We successfully developed a reproducible and efficient liquid-liquid extraction technique to extract imipramine and desipramine from plasma samples for HPLC analysis, with > 80 % extraction efficiency on average and very little variation between different extractions (Figure C**.3**). Therefore, using this optimised extraction procedure it was possible to determine plasma concentrations of imipramine and desipramine over time following intravenous imipramine administration, thereby generating pharmacokinetic profiles for imipramine and desipramine in both anaesthetized and conscious freely moving animals following imipramine delivery via various routes of administration.

Initial experiments were carried out on a non-recovery basis. Therefore, a plasma pharmacokinetic profile for imipramine and desipramine was firstly determined in an anaesthetized animal for 90 minutes post-intravenous imipramine administration (Figure C**.4**). In a subsequent experiment the intravenous catheter was not patent, meaning it was not possible to administer imipramine intravenously, but it was possible to sample blood via the intra-arterial catheter. Therefore, imipramine was administered intraperitoneally to the anaesthetized rat and blood samples were taken for 90 minutes following imipramine administration (Figure C**.5**). Once an adequate level of surgical proficiency was attained, rats were allowed to recover overnight prior to drug administration and blood sampling. A pilot experiment to help determine an acceptable dose was carried out in one animal which received an i.v. dose of imipramine 1 $mg.kg^{-1}$ (Figure C**.6**). This dose

was well-tolerated, and subsequent experiments used a higher imipramine dose of 5 $mg.kg^{-1}$ which was also well-tolerated. This 5 $mg.kg^{-1}$ i.v. dose was repeated in 6 animals, resulting in a typical intravenous drug concentration versus time plot for imipramine with low levels of variation at each time-point (Figure C**.7**). Furthermore, it was also possible to quantify desipramine levels in these plasma samples, thus yielding a plasma profile for desipramine in the conscious freely moving animal following imipramine administration. The concentration of desipramine in plasma gradually increased over time before declining, as was expected considering desipramine is a metabolite of imipramine.

Further experiments were carried out in dual-catheterized animals which involved the oral administration of imipramine. The aim here was to demonstrate that the plasma profile of imipramine would be different following administration via the oral route compared to the intravenous route. This was the case as illustrated in Figure C**.8**. The high levels of variability observed at each time point reflect the variable nature of drug absorption from the gut (particularly in non-fasted animals, as was the case here), and supports our decision to administer imipramine intravenously, thereby reducing the variability in plasma levels.

In addition to the determination of imipramine and desipramine plasma profiles after imipramine administration, we also determined plasma corticosterone levels in one dual-catheterized animal at various time-points on the day after catheterization surgery. The aim here was two-fold: firstly to demonstrate that we could detect the diurnal variation in plasma corticosterone levels using our catheterization technique and secondly to determine if the plasma corticosterone levels detected would be elevated relative to expected values. Corticosterone is the main stress hormone in rats, and circulating plasma corticosterone concentrations reflect the stress levels of the animal. As expected, we observed a diurnal variation in plasma corticosterone levels over the course of the day, with trough values observed in the morning shortly after turning the lights on and peak values in the evening shortly after turning the lights off (Figure C**.9**). This pattern is broadly in line with reports in the literature (Atkinson and Waddell, 1997). Furthermore, the mean plasma corticosterone concentration measured across the day (145.8 $ng.ml^{-1}$) was

within the expected range for an unstressed female rat (Atkinson and Waddell, 1997), thus indicating that the rat was not suffering from an increased level of stress on the day following catheterization surgery.

The present study also aimed to establish the intracerebral microdialysis technique. To this end, *in vitro* experiments were firstly undertaken which demonstrated that imipramine, desipramine, verapamil and trimipramine could be reliably and reproducibly recovered in our 'home-made' probes from wells containing a standard 100 $ng.ml^{-1}$ solution of these drugs (Figure C**.10**). The next step involved carrying out *in vivo* experiments to determine if imipramine and desipramine could be recovered from the brains of rats which had undergone microdialysis surgery. On the day after microdialysis surgery, imipramine (30 $mg.kg^{-1}$ i.p.) was administered to these animals and microdialysis samples were collected over time. It was possible to detect both imipramine and desipramine in all of these samples, thus yielding an intracerebral concentration versus time profile for these drugs following i.p. administration (Figure C**.11**). This demonstrates the success of our intracerebral microdialysis technique.

In conclusion, the present results demonstrate that it is possible to individually use our catheterization or microdialysis procedures, in combination with our HPLC-ECD analytical technique, to monitor fluctuations in imipramine and desipramine concentrations in plasma or the brain over time. The next step involves combining the two surgical procedures to monitor alterations in imipramine transport across the BBB in response to interventions such as P-gp inhibition.

Appendix D: Microdialysis probe construction

D.1. Background information

Microdialysis is a sampling technique used to collect small molecular weight substances from the extracellular fluid of the brain/spinal cord. Substances diffuse across a semi-permeable membrane down their concentration gradient. All microdialysis probes are composed of a length of tubular dialysis semipermeable membrane through which a solution, usually devoid of the analyte of interest (usually artificial cerebrospinal fluid), is constantly perfused. A variety of membranes are available which differ in pore size and the material used. Once the probe is inserted into the brain/spinal cord region of interest and perfusion begins, substances in the extracellular fluid diffuse through the membrane into the probe along their concentration gradient. The perfusate is infused slowly and continuously through the length of the dialysis probe into the outflow tubing where it can be collected for subsequent analyte quantification.

D.2. Materials

Polyethylene tubing (PE-10, PE-50)

Fused silica

Semipermeable membrane (choose specific molecular weight cutoff)

Scissors

Forceps

Needles (orange)

Epoxy adhesive glue-Epoxy Part A and Hardener Part B

Ruler

Microscope

A4 sheet (for mixing glue)

D.3. Step-by-step guide

D.3.1. Probe building

1. Cut PE-10 (16 inches and 10 inches), PE-50 (2 inches) and Silica (19 inches) and hang on upturned sticky tape. If old probes available, can reuse PE-10 for covering silica, and PE-50 if long enough.
2. Put PE-50 over 16 inch PE-10 (may need to use blue 23G needle to loosen PE-50). Glue in place.
3. Make hole in one side of PE-10 at the end which does not have PE-50 attached (~4cm from top) using orange needle. Hold needle in right hand and have long side of PE-10 on right so that hole made forms lip which glue cannot get into.
4. Know where the hole was made. Holding the PE-10 up with the hole facing up and slightly curved, feed silica in through short end of PE-10 and pull through hole. Leave a small bit of silica showing at the top of the PE-10.
5. Cut membranes (about 12-15mm so that long enough to tuck in). Always hold membrane with forceps, never with fingers. To place membrane onto silica place membrane on microscope stage. Hold the membrane near top using forceps without applying pressure, and push silica into the membrane. Tuck a good amount of membrane under PE-10 so that glue won't block it. Glue membrane in place. Measure membrane from end of glue spot to top of membrane under microscope. Want 6mm of membrane for prefrontal cortex, or 4.5mm for hippocampus, or otherwise as according to the literature, and chop off any excess using blade.
6. Place wet glue in membrane tip and allow capillary action to work so that ~0.75mm of the membrane is filled with glue on inside only.
7. Need to block of some of the membrane from the connection with PE-10 so that only a certain amount will contribute to the 'dialysing tip'. Active site for PFC=3mm, Hippocampus=2mm. Block off any remaining membrane using a very thin, smooth layer of glue. Mark the spot for blocking the membrane using fused silica.

8. Once top glue spot dry, push silica as close as possible to glue spot without touching (~0.2mm). Glue silica in place. Be very careful that the glue is not too runny at this stage as it may block the probe.
9. Attach 10 inch PE-10 over silica and glue in place.

D.3.2. Make aCSF (quantities for 500ml):

NaCl	147mM	4.29534g
KCl	4mM	0.1491g
$MgCl_2.6H_2O$	0.9mM	0.091485g
$CaCl_2$	1.7mM	0.094335g

Put 250ml HPLC grade water into 500ml glass bottle. Add salts. Make up to 500ml with HPLC grade water. Aliquot out by filtering using 0.2mcM filter attached to syringe. aCSF can be stored for a few weeks.

D.3.3. Activate membrane of the probe

Put ethanol in syringe (Terumo brand). Put blue needle into PE-50, attach syringe and push ethanol through finished probe to activate the membrane. Wait to see ethanol coming out the end of the silica-no blockages. Run aCSF through probe using a syringe and attach to pump running at 1.5µL/min with membrane immersed in medicine cup of aCSF (an activated membrane will shrink unless immersed in liquid).

D.3.4. Setting up syringes in pump.

Add filtered aCSF to 5ml syringe and mount on pump. End of barrel and end of insert should fit into gaps. Tighten side bars, then front, then back. Push out some aCSF and then click into place. Turn on, press run (1.5µL/min), make sure flow switch going the right way. Attach probes that are ready to it and immerse membrane in aCSF. Attach probe to orange frame and glue silica end below it.

Appendix E: Microdialysis surgery protocol

E.1. Background information

Microdialysis is a sensitive technique used to sample extracellular fluid for levels of drugs, drug metabolites, neurotransmitters, amino acids etc. It is renowned as a sensitive, versatile technique which has applications in elucidating neuropharmacology of drugs and diseases, as well as giving pharmacokinetic information on drug levels in the brain. While it was developed over 30 years ago in the 1970s and 1980s in Sweden, it has gained popularity as a useful and powerful sampling technique in recent times. This technique is known to hold many advantages over other available brain sampling techniques, including:

- ✓ Continuous sample collection from live, awake, freely-moving animals pre- and post- drug administration/other manipulation
- ✓ Measurement of free (unbound) drug concentrations = active form of drug
- ✓ Measurement of drug/neurotransmitter levels in a specific area of the brain- ability to measure levels in discrete regions
- ✓ Collection of clean samples, that do not require extra purification prior to analysis
- ✓ Potential application in wide range of species, from small rodents (mice, rats) to non-human primates

E.2. Materials

Stereotaxic frame
Scalpel
Clips
Cotton buds
Adrenaline 0.1%
Blue needle
Probe (calibrated)
Screws
Forceps
Screwdriver
Drill
Marker

Dental cement (powder and solvent)
Petri dish
Spatula
Spring
Needle and thread
Microdialysis cage
Allen keys
Bedding, Chow, Water bottle
Tape

E.3. Step-by-step guide

1. Set up rat on stereotaxic frame.
2. Make one incision from eyes to ears using scalpel.
3. Use 4 clips to pull back skin from four corners.
4. Use cotton buds to move away layer above skull.
5. Dip cotton buds in adrenaline and dab over skull to constrict vessels and stop bleeding.
6. This should allow time for bregma to appear.
7. Mark bregma with marker.
8. Take AP(anterior posterior) and ML (medial-lateral) co-ordinates.
9. Calculate co-ordinates of where you want to go to-hippocampus, PFC etc. based on co-ordinates in literature/rat brain atlas.
10. Move needle on stereotaxic frame to that point.
11. Mark skull with needle.
12. Use marker to mark spot also if required.
13. Mark two other spots to the back of the area for screws which lend support (this makes a triangle type area).
14. Drill all three holes (drill speed 4, only until drill pops back up). Make sure the microdialysis probe hole for entry is completely free of membrane by putting a needle in past skull to pop any remaining membrane.
15. Place screws in back two holes, holding with forceps and screwing with screwdriver (may require downward pressure).
16. Insert probe, making sure it enters center of the hole and is pointed straight downward.

17. As it is being lowered in, stop just when it is in past the level of the skull, and take DV (dorsal ventral) co-ordinates. Calculate how far into the brain the probe should be placed and lower it in.
18. Mix some cement and solvent in a petri dish and start to cement the probe in place, using the screws for support. This will require many layers of glue.
19. Place spring over probe and cement in place also.
20. Start stitching up wound on nape of neck (from cannulations) while waiting for cement to dry. Once cement is dry, stitch up head.

E.4. Post-surgery

Place rat onto bedding in special cage. Attach spring using allen key to loosen holding arm. Make sure spring is loose enough that rat can move around. Using tape tie down silica. Attach PE-50 to syringe pump outlet.

Appendix F: Jugular Vein Catheterisation

F.1. Methodological notes

1. Making taps for JV and CA catheterisation. Clamp needle near base by holding with needle holder and cut using scalpel.
   - Blue needle for JV
   - Orange needle for CA
2. Shave back of neck and top of chest (right hand side if rat lying with chest up and head towards you, legs away from you. Pull taught while shaving and shave with blade 'scooping'.
3. Give subcutaneous injection of Carprofen (100 µl Rimadyl in 5 ml saline). Pinch skin on back and inject ~0.8ml in bottom part of V.
4. Swab shaved areas with alcohol to sterilize and wipe away excess hair.
5. Make incision to right of centre along line of beating vein. Make incision using scissors. Holding skin with forceps (one arm inside and one out), pull subcutaneous tissue away from skin using another forceps. Make pouch below incision, pull away to right and small bit on left.
6. Butterfly using needle holder, to separate subcutaneous tissue more.
7. Make incision at back of neck using scissors. Pull subcutaneous tissue away from skin and tunnel to left using needle holder (pushing out against skin so as not to damage anything).
8. Pull JV catheter through using needle-holder, blue marked end (2.5 cm from tip of catheter) pointed toward vein side.
9. Insert catheter through 2 syringe barrels (to protect from chewing when rat regains consciousness). Attach 3-way tap with blue needle (with valve closed) to line and flush with 0.35ml-0.4ml saline.
10. Pull apart subcutaneous tissue with small forceps and be careful once near vein. Isolate vein carefully from sides by pulling away fat/subcutaneous tissue. When isolated, put forceps with arms facing up under JV so that JV rests in dip of forceps. Thread double piece of thread through, and cut into two pieces.

11. Tie of top piece of string tightly to slow blood flow. Leave arms long and clamp out of the way using needle holder.
12. Tie one very loose knot at the other end and attach clamp to it.
13. Make nick in side of vein using spring scissors, use tweezers to open up nick just enough to put catheter in. Hold catheter using forceps just above blue mark. Push into vein as far as blue mark and quickly tie the knot at bottom very tightl. Cut excess suture string away from knot.
14. Tie top piece of string around catheter and cut excess suture string away from knot. Check to see that line is patent by pushing in small bit of saline and drawing out and pushing back in small extra bit.
15. Sew up SC tissue top (leave arms of knot long to loop around catheter) and bottom (don't cut arms of knot). Make loop out of catheter and place into pouch. Loop bottom piece of string around catheter 3 or 4 times and tie knots them to secure in place. Check line is still patent and fill line with heparinised saline (0.4-0.45ml). Do the same on top. Sew up skin (mattress stitching works well).

Appendix G: Carotid Artery Catheterisation

G.1. Methodological notes

1. When catheterising both jugular vein and carotid artery, make incision at midline. Do jugular vein as per Appendix E. Try to ensure loop is pushed to the right so that carotid artery and jugular vein catheters are separated.
2. Pull jugular vein, barrels and taps over to the left-hand side so that carotid artery line can be threaded through barrels.
3. Start pulling apart subcutaneous tissue just to the left of midline. Do not go too near head.
4. Work through subcutaneous tissue until muscles with upside down 'V' in middle is visible. Holding muscle on left with small forceps, tease apart from V using other small forceps. Reach carotid artery and vagus nerve (together). Tease apart tissue to isolate carotid artery and vagus. Scoop up using small forceps.
5. Separate vagus from carotid. Using forceps make hole between carotid and vagus. Push against carotid rather than vagus. Insert small forceps into hole, and keeping pressure against carotid, separate carotid and vagus. Once carotid is free, clamp as close to the base as possible. Insert thread underneath and tie off at top. Throw loose knot in thread at bottom. Use needle holder on top thread to create tension on the vessel and drop over left hand side of head for correct angle.
6. Using spring scissors, cut carotid top and side. Use straight forceps to open and insert CA tubing. Have catheter so that dip is angled into vein. Push in as far as clamp. Tighten thread over artery and catheter. Remove clamp and insert catheter to blue line. Tie thread. Stitch up subcutaneous, make loop etc as described in relation to jugular vein.
7. Always check patency of catheters once in. Make sure to fill with saline before inserting into rat and heparinised saline after.