P-glycoprotein differentially affects escitalopram, levomilnacipran, vilazodone and vortioxetine transport at the mouse blood–brain barrier in vivo

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1. Introduction

A prerequisite to achieve therapeutic efficacy of a given drug is that there is a relevant exposure at the therapeutic target(s). In the central nervous system (CNS), active transporter-mediated efflux across the blood–brain barrier (BBB) is a well-known mechanism to extrude a broad range of exogenous compounds from the CNS. P-glycoprotein (P-gp, ABCB1, MDR1) is one of the major efflux transporters present in the luminal side of the BBB and is responsible for the complete avoidance of CNS exposure to strong substrate compounds (Pardridge, 2007; Schinkel, 1999; Urquhart and Kim, 2009). For many psychotropic compounds that are able to cross the BBB, brain concentrations in vivo can be reduced by P-gp to varying degrees in both animals and humans (Doran et al., 2005; Linnet and Ejsing, 2008; Syvanen et al., 2009). Several different classes of antidepressants have been shown to be P-gp substrates including the tricyclic antidepressants, selective serotonin (5-HT) reuptake inhibitors (SSRIs) and serotonin noradrenaline reuptake inhibitors (SNRIs). Accordingly, compounds such as amitriptyline, imipramine, citalopram, fluoxetine, fluvoxamine and venlafaxine display higher brain-to-plasma distribution ratios in P-gp knockout (KO) mice compared to wild-type (WT) counterparts (O’Brien et al., 2012). Thus, substrate promiscuity or polyspecificity is a renowned characteristic of P-gp and it has been shown that P-gp recognizes various chemically and structurally diverse substrates (Chufan et al., 2015). In the clinical setting, depressive states have been hypothesized to be linked to increased P-gp activity in medicated patients indicated from positron emission tomography.

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(PET) studies (de Klerk et al., 2009) although P-gp function in medicated depressed patients is far from fully elucidated.

In recent years it has, based on the above observations, been hypothesized that resistance to antidepressant treatment may be associated with polymorphism(s) within the ABCB1 gene encoding for P-gp, conjecturing that the polymorphism affects the P-gp mediated drug efflux at the BBB to a degree that prevents therapeutic concentrations being reached in the CNS. The hypothesis that allelic variation in the ABCB1 gene is associated with treatment outcome has been addressed in a number of clinical studies (for reviews, see (O’Leary et al., 2014; Ray et al., 2015; Rosenhagen and Uhr, 2011). The data have been conflicting, possibly due to differences in study designs, heterogeneous patient populations, inadequate statistical power, and polymorphic heterogeneity (O’Leary et al., 2014). Thus a recent meta-analysis including 2859 patients diagnosed with major depressive disorder and focusing on six ABCB1 single nucleotide polymorphisms (SNPs) indicated that a subset of these SNPs were associated with treatment outcome (Breitenstein et al., 2015). Hence, according to this paradigm, the affinity for P-gp at the BBB for a given drug may influence the variability in CNS bioavailability due to polymorphism linked to ABCB1. Furthermore, P-gp mediated efflux may also impact the side-effect profile since the peripheral drug exposure needs to be increased to obtain sufficiently high plasma concentrations to overcome P-gp mediated brain efflux. In addition to P-gp, the efflux transporter breast cancer resistance protein (Bcrp) may contribute to BBB efflux function. Reports with KO mice have indicated that these two transporters acts in a cooperative manner for some compounds implicating that absence of both P-gp and Bcrp results in a significantly larger effect than the combined effects from the single transporter KO mice (Agarwal et al., 2011; Kodaira et al., 2010).

Within the recent years new antidepressants have been introduced to the market including levomilnacipran, the levorotatory enantiomer of the SNRI milnacipran (Mago et al., 2014), the 5-HT1A receptor agonist and an inhibitor of the serotonin transporter (SERT) (Alvarez et al., 2014). These drugs are prescribed for in vivo brain distribution of these drugs implicating that absence of both P-gp and Bcrp results in a significantly larger effect than the combined effects from the single transporter KO mice (Agarwal et al., 2011; Kodaira et al., 2010). Thus a recent meta-analysis including 2859 patients diagnosed with major depressive disorder and focusing on six ABCB1 single nucleotide polymorphisms (SNPs) indicated that a subset of these SNPs were associated with treatment outcome (Breitenstein et al., 2015). Hence, according to this paradigm, the affinity for P-gp at the BBB for a given drug may influence the variability in CNS bioavailability due to polymorphism linked to ABCB1. Furthermore, P-gp mediated efflux may also impact the side-effect profile since the peripheral drug exposure needs to be increased to obtain sufficiently high plasma concentrations to overcome P-gp mediated brain efflux. In addition to P-gp, the efflux transporter breast cancer resistance protein (Bcrp) may contribute to BBB efflux function. Reports with KO mice have indicated that these two transporters acts in a cooperative manner for some compounds implicating that absence of both P-gp and Bcrp results in a significantly larger effect than the combined effects from the single transporter KO mice (Agarwal et al., 2011; Kodaira et al., 2010).

2. Materials and methods

2.1. Animals

Male mdrla-deficient mice (P-gp knock-out (KO) mice, Crl:CF1-Abcb1a<sup>+/−</sup> mutants) and CF-1 (non-Swiss) outbred background mice (wild-type (WT) mice) (18–22 g at arrival) were purchased from Charles River Laboratories (Wilmington, MA, USA). Male mdrla<sup>−/−</sup>Bcrp1 KO (P-gp/Bcrp KO, FVB:129P2-Ashc1a<sup>+/−</sup>Borc-Ashg2<sup>+/−</sup>Ahs) and WT FVB mice (18–22 g) were obtained from Taconic (Germantown, NY, USA). Animals were housed individually in a temperature (21 ± 1 °C) and humidity (40–50%) controlled environment, with lighting maintained under a 12 h light–dark cycle, and were habituated for at least one week prior to experimentation. Food and water were available ad libitum. Ethical permission for the procedures used in this in vivo study was granted by the Danish Animal Experiments Inspectorate, and all animal procedures were performed in compliance with Directive 2010/63/EU of the European Parliament and of the Council, and with Danish Law and Order regulating animal experiments (LBK no 253, 08/03/2013 and BEK no 88, 30/01/2013).

2.2. Drugs and reagents

Escitalopram oxalate, vilazodone hydrochloride and vortioxetine hydrobromide were synthesized at H. Lundbeck A/S. Levomilnacipran hydrochloride was obtained from Sigma–Aldrich (St. Louis, MO, USA). [3H]DASB (N,N-dimethyl-2,2-amino-4-cyanophenyl-thiobenzylamine, specific activity 80 Ci/ mmol) was purchased from American Radiolabeled Chemicals, (Saint Louis, MO, USA). All other chemicals were high performance liquid chromatography or reagent grade and were obtained from Sigma–Aldrich.

2.3. Acute brain distribution studies

Each test compound was dosed subcutaneously (sc) to groups of P-gp KO and WT mice. Escitalopram and vilazodone were dosed at 1 mg/kg, levomilnacipran at 2 mg/kg and vortioxetine at 0.5 mg/kg (all as free base). The dose of each drug was selected to cover pharmacologically relevant systemic exposures and to provide sufficient data points to characterize the concentration-time courses. All dosing solutions were freshly prepared in 10% hydroxypropyl-β-cyclodextrin (pH 4) and administered in a volume of 10 ml/kg. Brain and plasma samples were obtained 0.25, 0.5, 1, 2 and 3 h after drug administration. In addition, brain and plasma exposure of levomilnacipran was measured in mice deficient of both P-gp and Bcrp (P-gp/Bcrp KO) and WT mice 1 h after sc dosing of 2 mg/kg. At the time of sampling, cohorts of 3 KO and WT mice were anesthetized by isoflurane followed by blood collection by heart puncture using heparinized syringes. Subsequently, animals were sacrificed; the brains were rapidly removed and gently rinsed on filter paper. All blood samples were centrifuged for 10 min at 5000 g at 4 °C, and the harvested plasma and brain samples was stored at –80 °C until bioanalysis.

2.4. Steady-state brain distribution studies

Continuous dosing to steady-state was performed by sc implantation of osmotic minipumps (Alzet 2001, 1.0 μl/h, Durect Corporation, Cupertino, CA) into groups of P-gp KO and WT mice. Pumps were loaded with drug solutions prepared in hydroxypropyl-β-cyclodextrin (pH 4) for all test compounds. The following concentrations (as free base) with corresponding average doses calculated on the basis of body weight and pump delivery rate were applied: escitalopram 6 mg/ml (5 mg/kg/day), levomilnacipran 15 mg/ml (14 mg/kg/day), vilazodone 20 mg/ml (19 mg/kg/day), vortioxetine 0.75 mg/ml (0.7 mg/kg/day). The doses were selected to target steady-state plasma concentrations inside the concentration ranges in the acute time-course studies. After loading the test compounds, the pumps were primed overnight in sterile saline at 37 °C. Pumps were implanted sc under aseptic conditions on the backs of the animals as described previously (Bundgaard et al., 2012). For each test compound, a group size of 4–6 P-gp KO and WT mice were used. After 72 h of continuous drug release, animals were euthanized, and brain and blood
samples were harvested as described above.

2.5. In vivo occupancy studies

In vivo cortical SERT occupancy was measured in groups of P-gp KO and WT mice receiving 4–5 different acute sc doses of vilazodone in the range of 0.01–1 mg/kg (n = 3/group). Occupancies were measured 30 min after dosing of vilazodone. The radioligand ([3H]DASB) was injected intravenously as a bolus dose of 2.9 µCi/mouse 15 min after administration of vilazodone. The animals were killed by decapitation, the brains quickly removed, and the cortex dissected out and homogenized in 5 ml ice-cold buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, pH 7.4). Homogenate (1.0 ml) was filtered through 0.1% PEI-soaked Whatman GF/C filters. This was completed within 60 s subsequent to decapitation. Filters were washed twice with 5 ml ice-cold buffer and counted in a scintillation counter. A group of animals treated with 2.0 mg/kg paroxetine was used to determine full occupancy corresponding to nonspecific [3H]DASB binding. A group of vehicle-treated animals (10% hydroxypropyl-β-cyclodextrin, pH 4) was used to determine total [3H]DASB binding. Cardiac blood samples were taken from all animals during the occupancy study (30 min after dosing of vilazodone) and plasma was analyzed for concentrations of vilazodone.

2.6. Bioanalysis

Samples were prepared by homogenizing the brain tissue using isothermal focused acoustic ultrasonication (Covaris E220x, Covaris, Inc., Woburn, MA). To each whole brain, deionized water (Millicel, Inc., Woburn, MA) was added (1:4 w/v) and samples were processed for 3.5 min at a bath temperature of 7–10 °C (1000 cycles per burst, duty cycle 50%). 100 µl homogenate was transferred to 96-well plates, sealed and stored at –80 °C. At the day of analysis, brain homogenates and plasma samples were precipitated with acetonitrile containing internal standard. For escitlamipran and vortioxetine their corresponding stable isotope-labeled 13C6-version was used as internal standard. For levomilnacipran and vilazodone a generic internal standard was used (Lu AE90074). Quantitative analyses were performed using ultra-performance liquid chromatography (Acquity UPLC system; Waters, Milford, MA) followed by tandem mass spectrometry detection (Sciex-API 4000 mass spectrometer, AB Sciex, Foster City, CA). The lower limit of quantification was 0.5 ng/ml in plasma and 1 ng/g in brain for all test compounds (peak signal/noise ratio >5).

2.7. Data analysis

The area under the mean composite total plasma and brain concentration–time curves (AUCs) after acute dosing were calculated using GraphPad Prism (GraphPad, San Diego, CA). All AUCs were calculated from time zero to last point of measurement which corresponded to 3 h for all test compounds in both plasma and brain. The total brain to plasma distribution after acute dosing (Kp,uu) was calculated as AUCbrain/AUCplasma. Following continuous dosing to steady-state, the mean total brain to plasma distribution (Kp,ss) was calculated. The total concentrations were subsequently converted to unbound concentrations by multiplying with the free fractions (fu) in the respective matrices obtained by equilibrium dialysis (Redrobe et al., 2014). The resulting ratio between unbound brain and unbound plasma concentrations at steady-state was calculated for each animal and denoted Kp,un (Gupta et al., 2006). A two-tailed Student’s t-test for unpaired observations was used to compare differences between groups. P-values <0.05 were considered statistically significant. Individual mean comparisons during time-course analyses after acute dosing were corrected for multiple testing by the Bonferroni correction. A critical value of 0.01 was calculated by dividing the familywise error rate (0.05) by the number of tests (5). Plasma concentrations resulting in 50% in vivo SERT occupancy (EC50) was calculated from plasma concentration–response curves in P-gp KO and WT mice based on nonlinear regression using an Emax model (occupancy = [Emax x C^n]/[EC50 + C^n]), where C is plasma concentration and n is the sigmoidicity of the curve. Minimum and maximum occupancies were constrained at 0 and 100% respectively, for the model fitting (GraphPad, San Diego, CA).

3. Results

Mean composite brain and plasma concentration–time courses and the corresponding calculated Kp-values after an acute sc injection are shown in Fig. 1. For all drugs and for both genotypes brain and plasma concentrations were captured over the entire 3 h time-course. Compared to WT mice, brain concentrations in the KO mice were significantly higher at all measured time-points for escitalopram, levomilnacipran and vilazodone, whereas vortioxetine exhibited slightly higher but statistically insignificant brain concentrations in KO mice at all measured time-points. In plasma, escitalopram and levomilnacipran levels coincided over the full time-course, whereas vilazodone showed slightly higher plasma concentrations in WT mice, with statistically significant differences between KO and WT mice at 1 h. Vortioxetine generally showed slightly higher plasma concentrations in KO mice versus WT, and reached statistical significance between genotypes at 1 h.

The Kp-values increased over time for all drugs in both genotypes. The increase in Kp was continuous over the duration of the study in WT mice, whereas in KO mice, escitalopram and vilazodone had reached a plateau at the terminal time-point, when a pseudo distributional equilibrium between plasma and brain had been attained. The aggregate brain and plasma exposure AUCs for the drugs and their corresponding Kp,uu-values are summarized in Table 1. From the ratio between Kp,uu in KO and WT mice, the brain distribution enhancement ratio was calculated, taking both time dependency and differences in plasma concentrations between genotypes into account. As shown in Table 1, vortioxetine had an enhancement ratio very close to 1, whereas escitalopram, vilazodone and levomilnacipran respectively showed enhancement ratios in their brain distribution of approximately 3, 5 and 6, which suggest a P-gp mediated efflux at the BBB level for these 3 antidepressants.

The plasma and brain concentrations obtained in P-gp KO and WT mice at steady-state following 3-days of continuous treatment by osmotic minipumps are shown in Fig. 2. Brain concentrations of escitalopram, levomilnacipran and vilazodone were significantly higher in KO mice compared to their WT counterparts, with enhancement ratios in brain exposure of 2.6, 7.7 and 2.5, respectively. Brain levels of vortioxetine displayed an enhancement ratio of 1.3 in KO mice versus WT mice, but was not statistically different from levels in WT mice. For all tested drugs, plasma concentrations were somewhat lower in KO mice compared to WTs although not reaching statistical significance for any of them (Fig. 2). The calculated brain-to-plasma distribution ratios at steady-state expressed as total (Kp,ss) and unbound (Kp,uu) values are summarized in Table 2. The brain distribution enhancement ratios (Kp,uu) for escitalopram, vilazodone and vortioxetine were in the same range as observed after acute dosing whereas an enhancement ratio of 9 was observed for levomilnacipran at steady-state likely reflecting the lack of equilibrium in the acute setting where an enhancement ratio of 6 was achieved.

To further evaluate the BBB distribution and P-gp mediated efflux in quantitative terms, the Kp,uu was calculated at steady-state in P-gp
Fig. 1. Brain (left panel) and plasma (middle panel) composite drug concentration-time profiles in P-gp KO and WT mice following sc dosing of escitalopram (1 mg/kg, a), levomilnacipran (2 mg/kg, b), vilazodone (1 mg/kg, c) and vortioxetine (0.5 mg/kg, d). The derived brain-to-plasma distribution ratios (Kp) at each time-point are shown in the right panel. Each data point is presented as mean ± SEM (n = 3). *p < 0.05 between KO and WT value at each time-point corrected for multiple comparisons.
KO and WT mice (Table 2). This parameter expresses the net result of passive and active transport of the unbound drug across the BBB with a value of unity reflecting similar influx and efflux clearance across the BBB (Hammarlund-Udenaes et al., 2008). The brain distribution of levomilnacipran was found to be extensively restricted in WT mice, with a Kp,uu value of 0.038. This value was increased to 0.37 in mice lacking P-gp, indicating a substantial role of this transporter in limiting its brain penetration, though only a partial role as it remained well below unity in KO animals. To assess a potential contributing role of breast cancer resistant protein (Bcrp) in the active brain flux seen for levomilnacipran, brain and plasma exposure of this drug was also measured in P-gp/Bcrp KO and WT mice and compared to exposures in mice lacking only P-gp (Table 3). Drug concentrations were measured 1 h after dosing corresponding to the time of maximum brain levels seen in the time-course studies.

Fig. 2. Brain and plasma drug concentrations in P-gp KO and WT mice after continuous dosing over 3-days via osmotic minipumps (escitalopram 5 mg/kg/day, levomilnacipran 14 mg/kg/day, vilazodone 19 mg/kg/day, vortioxetine 0.7 mg/kg/day). Data are shown as mean ± SEM (n = 4–6). *p < 0.05 versus corresponding WT concentration.

Table 2
Steady-state brain-to-plasma distribution ratios expressed as total (Kp,ss) and unbound (Kp,uu) drug in P-gp WT and KO mice after a constant subcutaneous infusion via osmotic minipumps for 72 h. Data are presented as the mean ± SEM (n = 4–6 in each group).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kp,ss WT</th>
<th>Kp,ss KO</th>
<th>Kp,ss KO/WT</th>
<th>Free fraction (%)</th>
<th>Kp,uu WT</th>
<th>Kp,uu KO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUCplasma</td>
<td>AUCbrain</td>
<td></td>
<td>fu,plasma</td>
<td>fу,brain</td>
<td></td>
</tr>
<tr>
<td>Escitalopram</td>
<td>3.2 ± 0.3</td>
<td>10 ± 0.6</td>
<td>3.1</td>
<td>32</td>
<td>3.5</td>
<td>3.1 ± 0.03</td>
</tr>
<tr>
<td>Levo</td>
<td>0.14 ± 0.01</td>
<td>1.3 ± 0.1</td>
<td>9.3</td>
<td>77</td>
<td>21</td>
<td>0.37 ± 0.03</td>
</tr>
<tr>
<td>Vilazodone</td>
<td>0.88 ± 0.09</td>
<td>4.3 ± 0.55</td>
<td>4.9</td>
<td>1.3</td>
<td>0.40</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Vortioxetin</td>
<td>1.2 ± 0.3</td>
<td>1.7 ± 0.1</td>
<td>1.4</td>
<td>0.40</td>
<td>1.2 ± 0.3</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

KO and WT mice (Table 2). This parameter expresses the net result of passive and active transport of the unbound drug across the BBB with a value of unity reflecting similar influx and efflux clearance across the BBB (Hammarlund-Udenaes et al., 2008). The brain distribution of levomilnacipran was found to be extensively restricted in WT mice, with a Kp,uu value of 0.038. This value was increased to 0.37 in mice lacking P-gp, indicating a substantial role of this transporter in limiting its brain penetration, though only a partial role as it remained well below unity in KO animals. To assess a potential contributing role of breast cancer resistant protein (Bcrp) in the active brain efflux seen for levomilnacipran, brain and plasma exposure of this drug was also measured in P-gp/Bcrp KO and WT mice and compared to exposures in mice lacking only P-gp (Table 3). Drug concentrations were measured 1 h after dosing corresponding to the time of maximum brain levels seen in the time-course studies.

Table 3
Brain and plasma concentrations of levomilnacipran in P-gp and P-gp/Bcrp KO and WT mice and their corresponding Kp values 1 h after sc dosing of 2 mg/kg. Kp KO/WT denotes the enhancement ratio obtained in each genotype relative to WT. Data are presented as mean ± SEM (n = 3).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Brain (ng/g)</th>
<th>Plasma (ng/ml)</th>
<th>Kp</th>
<th>Kp KO/WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp KO</td>
<td>354 ± 63</td>
<td>259 ± 59</td>
<td>1.4 ± 0.09</td>
<td>5.0</td>
</tr>
<tr>
<td>P-gp WT</td>
<td>55 ± 3.7</td>
<td>200 ± 1.2</td>
<td>0.28 ± 0.02</td>
<td>4.7</td>
</tr>
<tr>
<td>P-gp/Bcrp KO</td>
<td>259 ± 12</td>
<td>291 ± 15</td>
<td>0.89 ± 0.07</td>
<td>4.7</td>
</tr>
<tr>
<td>P-gp/Bcrp WT</td>
<td>46 ± 6.6</td>
<td>246 ± 27</td>
<td>0.19 ± 0.04</td>
<td>4.7</td>
</tr>
</tbody>
</table>

in P-gp KO and WT mice (Fig. 1). Comparable enhancement ratios of around 5 were observed in both pairs of genotypes. Thus, no additional enhancement in the brain penetration of levomilnacipran was observed in mice with deletion of both Bcrp and P-gp compared to
P-gp alone. Escitalopram and vilazodone showed moderate transport restriction in WT mice at steady-state with $K_{p,uu}$ values around 0.3 which were both increased to approximately unity in KO mice. The unbound brain distribution of vortioxetine was found to be unrestricted in WT mice with a $K_{p,uu}$ close to unity, which was slightly higher in KO mice (Table 2).

The functional role of P-gp mediated efflux at the BBB was assessed by measurement of central SERT occupancy in vivo in P-gp KO and WT mice after different acute doses of vilazodone. In both genotypes, a concentration-dependent target displacement was obtained (Fig. 3). In line with the P-gp limiting effects on brain penetration, higher plasma concentrations of vilazodone were required in WT mice to achieve the same SERT occupancies as in KO mice. Accordingly, curve fits of the concentration-occupancy data using $E_{\text{max}}$ models resulted in EC50 values of 120 and 6 ng/ml in WT and KO mice, respectively.

4. Discussion

An increasing number of studies investigating the potential link between SNPs within ABCB1 and treatment response to antidepressant drugs have emerged over the recent years (Breitenstein et al., 2015; O’Leary et al., 2014). Assessment of the effect of P-gp on drug brain bioavailability is therefore important in order to put clinical pharmacogenetic data into context and to understand the pharmacokinetic/pharmacodynamic (PK/PD) properties of antidepressant drugs. Previous preclinical investigations have suggested that P-gp limits the delivery of several older antidepressant agents into the brain (Linnet and Ejsing, 2008; O’Brien et al., 2012). In this study, the P-gp mediated brain efflux of the newly introduced antidepressants levomilnacipran, vilazodone and vortioxetine was evaluated in vivo and compared to escitalopram using P-gp KO and WT mice after acute dosing and continuous treatment to steady-state.

The results of our study provided evidence that in mice, P-gp at the BBB impacted the CNS accessibility of these new antidepressants to different degrees. Thus, based on the brain-to-plasma enhancement ratios between genotypes, levomilnacipran was the strongest P-gp substrate after both single dose and continuous treatment followed by vilazodone and escitalopram, whereas vortioxetine was almost devoid of P-gp mediated efflux. The propensity of levomilnacipran for P-gp mediated efflux has also been shown in vitro in bidirectional transport studies in Caco-2 cells where a high efflux ratio was reported (ratio > 7) (Dyck et al., 2008).

Similar findings have also been reported for milnacipran (the racemic mixture containing the levo enantiomer) in Madin–Darby Canine Kidney cells expressing human P-gp (MDR1-MDCK cells, efflux ratio 23) (Van Orden et al., 2013). Efflux data with cells transfected with murine P-gp (Mdr1a) are not available in the literature so a direct in vitro-in vivo comparison within the mouse species cannot be made. The remarkable restriction in the brain distribution of levomilnacipran was also evident from the low $K_{p,uu}$ of 0.038 in P-gp competent WT mice. By calculating the unbound forms through $K_{p,uu}$, the BBB transport properties were thus separated from protein binding in plasma and binding to brain constituents. Interestingly, although $K_{p,uu}$ was increased almost 10-fold in P-gp KO mice to 0.37, the value was still substantially lower than unity in this genotype. If P-gp were the sole contributing efflux mediator, $K_{p,uu}$ should be around unity in mice devoid of P-gp activity. Hence, these results may indicate that additional transporter proteins besides P-gp are involved in the brain efflux of this drug in mice. To this end, it has been reported that for a range of substrates, brain distribution is influenced by both P-gp and Bcrp acting in concert at the BBB (Agarwal and Elmquist, 2012; Agarwal et al., 2011). However, for levomilnacipran, no additional increase in brain penetration was seen in P-gp/Bcrp KO mice compared to mice only devoid of P-gp. Thus, other hitherto unidentified transporter proteins are suggested to be involved in the efflux of levomilnacipran in mice in addition to P-gp.

Escitalopram and vilazodone were also found to be P-gp substrates in vivo with P-gp KO/WT enhancement ratios of about 3 and 5, respectively, for both acute dosing and at steady-state. For escitalopram, these results are similar to earlier findings in mice after both acute and repeated dosing (Karlsson et al., 2013) and in line with in vitro bidirectional MDCK cell transport studies where escitalopram was identified as P-gp substrate (efflux ratio 7) (O’Brien et al., 2013). The $K_{p,uu}$ values were around 0.3 in WT mice for both escitalopram and vilazodone suggesting a moderate net efflux which was ameliorated in KO mice which had $K_{p,uu}$ values around unity. Thus, for escitalopram and vilazodone, P-gp appears to be the dominating contributor to the active efflux in WT mice. To the best of our knowledge there are no published studies assessing vilazodone with respect to P-gp-mediated efflux at the BBB, neither in vitro nor in vivo. Here, we showed that P-gp substantially limited the brain exposure of vilazodone in mice. Thus, attainment of relevant brain concentrations and subsequent central target engagement would be dependent on P-gp activity. The results from in vivo occupancy of SERT, which is a key target for the mode of action of vilazodone, showed a dose- and concentration dependent binding in P-gp competent WT mice. This indicates that P-gp efflux does not preclude a central target binding in rodents, which is in line with other preclinical SERT occupancy data and pharmacological reports for vilazodone (Dawson and Watson, 2009; Hughes et al., 2005). However, a substantial leftward shift of the concentration–occupancy relationship was observed in P-gp KO mice, resulting in a 20-fold increase in potency based on the respective EC50 values (Fig. 3). This clearly shows that regulation of brain bioavailability via P-gp at the BBB can influence the functional response to vilazodone. In an analogous approach, the impact of chemically modulating P-gp activity has been described for escitalopram where pretreatment with the P-gp inhibitor verapamil enhanced the response to escitalopram in a behavioral assay for antidepressant-like activity (O’Brien et al., 2013). However, the same group recently reported that another P-gp inhibitor, cyclosporin A, did not augment the pharmacological effects of escitalopram measured as brain extracellular 5-HT in rats or at the behavioral level in mice despite a concomitant increase in escitalopram levels in the brain (O’Brien et al., 2014). Since both co-treatment with P-gp inhibitors and use of genetically modified

![Fig. 3. In vivo serotonin transporter (SERT) occupancy of vilazodone in the cortex of P-gp KO and WT mice, measured 30 min after sc dosing (0.01–1 mg/kg). Data points are expressed as mean percent occupancy ± SEM (n = 3 in each group). The curves represent non-linear fits of the concentration–occupancy relationships using an $E_{\text{max}}$ model ($E_0$ and $E_{\text{max}}$ being constrained to 0 and 100% respectively).](image-url)
animals may interfere with the phenotypic behavior, modulatory direct assays such as \textit{in vivo} target occupancy may be a more suitable approach to assess the functional P-gp effects rather than mechanistic behavioral studies. 

Among the four antidepressants tested, vortioxetine was found to be the weakest P-gp substrate \textit{in vivo} in mice. After acute dosing, the slight increase in brain exposure in KO mice compared to WT was followed by a similar increase in plasma AUC, resulting in comparable K\textsubscript{pu} values between genotypes. At steady-state, a modest increase in brain distribution was observed in P-gp deficient mice with a KO/WT enhancement ratio of 1.4. K\textsubscript{pu} was close to unity in WT mice and the modest effect of P-gp increased the K\textsubscript{pu} to 1.7 in KO mice. Although K\textsubscript{pu} values > 1 would normally be indicative of active uptake across the BBB, the strong binding of vortioxetine to plasma proteins and brain tissue (I\textsubscript{b} – 0.2% in plasma and brain tissue) makes the quantitative interpretation of whether K\textsubscript{pu} diverts from unity somewhat uncertain. The weak interaction of vortioxetine with P-gp \textit{in vivo} observed in the current experiments is in line with the low efflux ratio (<2) shown in transport studies in MDCK cells (Bundgaard et al., 2015). 

Comparison of the single dose derived composite brain and plasma concentration-time profiles with the steady-state distribution data reveals important aspects about the rate and extent of the distributional effects related to P-gp mediated efflux. For all four antidepressants, the K\textsubscript{pu} value increased over time in both genotypes after acute administration. For all identified P-gp substrates, the increase over time was more pronounced in KO mice than in WT. The largest time-dependent effect was seen with levomilnacipran, for which the K\textsubscript{pu} value increased by a factor of 26 from 0.27 at 15 min to 7.0 at 3 h in KO mice and by a factor of 14 in WT mice (0.08 at 15 min versus 1.13 at 3 h). Thus, using only a single time-point after acute dosing for the \textit{in vivo} evaluation of brain-to-plasma partitioning in general and quantification of distributional efflux-mediated effects in particular may be misleading, depending on the time-point chosen and the temporal differences in the distribution kinetics of the compound under investigation. These conclusions have also been pointed out in other CNS distribution studies with other drugs (Oberoi et al., 2013). Extracting the brain distribution information from the entire time-course by using the brain and plasma AUCs to derive the K\textsubscript{p} values is a common alternative to steady-state studies to assess the extent of brain penetration at distributional pseudo steady-state. In our experiments, the AUC-derived K\textsubscript{p} values were all within 3 times the K\textsubscript{p} values obtained at steady-state within each genotype. Furthermore, the KO/WT K\textsubscript{p} enhancement ratios obtained acutely from AUCs and those obtained at steady-state were in good agreement with a <2-fold difference for all drugs. Thus, consistent with earlier reports, the results indicate that AUC-derived brain/plasma partitioning data should be useful surrogates for the steady-state inter-compartmental distribution, also for P-gp substrates (Doran et al., 2012; Loryan et al., 2014).

The results presented here offer an insight into the role of P-gp in the brain distribution of the tested antidepressants in mice. How P-gp interactions in rodents translate to human is still an open question, since uncertainty exists concerning species differences and their possible significance. Assuming that substrate affinities for P-gp are generally conserved between species as shown for many compounds (Chu et al., 2013; Feng et al., 2008), an impact on brain bioavailability seen in rodents may pertain to humans. As a result, the variable expression or activity of P-gp caused by SNPs in the P-gp encoding genes in humans could alter the extent of brain distribution of compounds that are substrates for P-gp. By measuring drug concentrations in cerebrospinal fluid in humans as an indicative measure of brain exposure, such pharmacogenetic associations have been reported in genotyped patients in a small clinical study with citalopram (Nikisch et al., 2008). However, there are also reports in the literature indicating that species differences between human and mouse exists for some P-gp substrates (Katoh et al., 2006; Yamazaki et al., 2001). Also, P-gp protein expression in brain micro vessels from humans as well as monkeys has been reported to be approximately 3-fold lower than in mice (Ito et al., 2011; Kamiie et al., 2008; Uchida et al., 2011). This may translate to a varying degree of brain efflux depending on species suggestive of a diminished effect in higher species compared to mice. In rats, the influence of P-gp on brain uptake of different substrate drugs including risperidone and 9-hydroxyrisperidone has been shown to be of a similar magnitude as in mice (Bundgaard et al., 2012). In dogs and cynomolgus monkeys however, the K\textsubscript{pu} of these two drugs have been reported to be approximately 5 times higher in these species compared to rats (Doran et al., 2012). Interestingly though, the K\textsubscript{pu} of 9-hydroxyrisperidone in dogs and monkeys was 0.2–0.3 indicating a retained restriction in the BBB transport for this substrate drug also in these higher species. To allow for direct in vivo comparison between preclinical species and humans, a PET imaging study has been reported where three radiolabeled P-gp substrates was shown to have higher brain distribution in humans compared to rodents (Syvanen et al., 2009). This would support the notion of a diminished effect in higher species including humans although a potential net effect from P-gp at the human BBB cannot be ruled out. Even though considering that a direct quantitative translation cannot be made between rodents and humans, P-gp mediated effects in rodents will, due to the distribution disequilibrium, add another layer of complexity in the translation to the clinical setting. Thus, predictions of human efficacious plasma levels, doses and therapeutic indices for CNS drug candidates are often based on rodent data during the drug discovery process (Di et al., 2013). In addition, during that phase, the risk of having a situation where much higher systemic unbound concentrations relative to that in brain are needed to achieve the desired efficacy is deemed less attractive as this could lead to potentially unwanted effects owing to high systemic exposure.

In conclusion, the results of this study provided evidence that in mice, P-gp at the BBB impacted the \textit{in vivo} CNS accessibility in a group of newer antidepressants to different degrees. Levomilnacipran exhibited the most pronounced efflux liability followed by vilazodone whereas vortioxetine was almost devoid of P-gp mediated efflux from the CNS in mice. These results may help to facilitate informed interpretations of preclinical PK/PD studies as well as future clinical investigations of possible links between SNPs in ABCB1 and therapeutic response to the different newer antidepressants.

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