Utility of small-animal positron emission tomographic imaging of rats for preclinical development of drugs acting on the serotonin transporter

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Abstract

Visualization of neurotransmission components in living small animals using positron emission tomography (PET) has the potential of contributing to the preclinical development of neuroactive drugs, although it is yet to be examined whether quantitative animal PET data on candidate compounds can be extrapolated to humans. Here, we investigated the comparability of the occupancies of serotonin transporter (5-HTT) by therapeutic agents in rat PET studies with our predetermined data from ex-vivo animal experiments and clinical PET scans. Rats were treated with varying doses of fluvoxamine and a newly developed compound, (2S)-1-[4-(3,4-dichlorophenyl)piperidin-1-yl]-3-[2-(5-methyl-1,3,4-oxadiazol-2-yl)benzol[f]furan-4-yloxy]propan-2-ol monohydrochloride (Wf-516), and underwent PET scans with [11C]3-amino-4-(2-dimethylaminomethyl-phenylsulfanyl)-benzonitrile ([11C]DASB), a selective radioligand for in-vivo quantification of 5-HTT. PET images indicated a reduction of [11C]DASB binding to 5-HTT as a function of the doses and/or plasma concentrations of fluvoxamine and Wf-516. The doses of these drugs at half-maximal effect (15.2 mg/kg and 3.1 mg/kg, respectively), determined that using binding potentials for [11C]DASB, were comparable to those estimated by our previous ex-vivo measurements in rats (4.5 mg/kg and 1.1 mg/kg, respectively), as there was only a 3-fold difference between these results. Moreover, the plasma concentration of fluvoxamine needed for 50% occupancy of central 5-HTT (6.1 ng/ml) was almost equivalent to the value determined in human PET studies (4.6 ng/ml). These findings support the view that the conjunctive use of small-animal PET and [11C]DASB facilitates a quantitative comparison of in-development drugs targeting 5-HTT with established inhibitors and a predictive estimation of their plasma concentrations exerting therapeutic effects in humans.

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Introduction

The application of positron emission tomography (PET) to preclinical research and development of new therapeutic agents for treating neuropsychiatric disorders has become a subject of growing interest for neurobiologists as well as pharmaceutical manufacturers, since it permits a series of kinetic investigations of test compounds for the same individuals in non-invasive and fairly efficient manners. Imaging-based objective measures for the efficacy of drugs could be particularly indispensable requisites for the development of compounds alleviating symptoms of depression, schizophrenia and other mental disorders, in light of the fact that various animal models of these
conditions used for screening of therapeautants (Arguello & Gogos, 2006; Deussing, 2006; McArthur & Borsini, 2006) may not necessarily recapitulate the pathophysiology in humans (Duyk, 2003; Hurko & Ryan, 2005; Rupnias, 2003), and thus the therapeutic effects demonstrated in these models need justification on a pharmacodynamic basis.

Utilization of the in-vivo PET system in humans offers a practical means for estimating the occupancy of receptors and transporters for neurotransmitters by drugs targeting these biological components (Farde et al. 1998; Kapur et al. 2000; Meyer et al. 2004; Suhara et al. 2003; Takano et al. 2004, 2006). In addition, tracking the dynamic biodistribution of injected chemicals on maps of living animal brains has been made possible by high-resolution PET scanners dedicated to small-sized mammals (Tai et al. 2005; Yang et al. 2004). This current status has led to the expectation that in-vivo pharmacological indices, such as occupancy of target binding sites by exogenous agents, can be commonly determined by PET and the same radioligand across species, and would consequently span the gap between preclinical and clinical insights (Fowler et al. 1999; Wang & Maurer, 2005). Moreover, repetitive PET scans for each individual allows high-accuracy data acquisition with internal baseline controls (Herschman, 2003), providing obvious advantages over ex-vivo assays.

These notable benefits notwithstanding, it has also been documented that quantitative assays of molecular interactions between ligands and target binding sites should be conducted with great care by taking the ‘mass effects’ on the tracer kinetics into account. These effects primarily stem from the requirement of a high-dose radioligand administration to rodents with small body mass for acquisition of sufficient radiosignals, resulting in a large mass of injected compound. This also raises the occupancy of the targets by the tracers to a significant level. In a regular experimental protocol for rat PET study using [11C]raclopride, ~20% of dopamine D2 receptors were occupied by unlabelled ligand (Hume et al. 1998; Kung & Kung, 2005). Accordingly, specific binding of the radioligand to brain tissue might become markedly susceptible to subtle changes in its dose, specific radioactivity, metabolism and uptake/binding to peripheral organs. The latter two biological factors are likely to be variable among species, and be additionally influenced by pretreatment of animals with a non-radioactive test drug, even if a sufficiently long interval between its administration and injection of PET ligand is given. Additionally, the animals are obliged to be in an immobilized state by anaesthetics or specially made restraint devices during PET scans, which is unlikely to retain a normal physiological condition. Because these issues may preclude the simplicity of preclinical PET studies in a manner depending on the biomolecule of interest and the pharmacological properties of the applied radioligand, the feasibility of small-animal PET measurements aimed at obtaining quantitative data translatable to humans needs to be carefully examined for each individual experiment contemplated.

In this study, we measured the occupancy of central serotonin transporter (5-HTT) by (2S)-1-[4-(3,4-dichlorophenyl) piperidin-1-yl]-3-[2-(5-methyl-1,3, 4-oxadiazol-2-yl)benzo[b]furan-4-yloxy]propan-2-ol monohydrochloride (WF-516), a novel investigational antidepressant with a high affinity for serotonin transporter (5-HTT) and serotonin 1A (5-HT1A) receptor (El Mansari & Blier, 2008; Maeda et al. 2006a,b), by high-resolution PET imaging of rat brains with [11C]-3-amino-4-(2-dimethylaminomethyl-phenylsulfanyl)-benzonitrile ([11C]DASB). [11C]DASB was developed as a specific radioligand suitable for PET imaging of 5-HTT by in-vitro/ex-vivo (Wilson et al. 2000) and in-vivo (Huang et al. 2002) comparative evaluations of a series of candidate and pre-existing compounds, and was shown to permit accurate PET quantification of 5-HTT in living brains (Ginovart et al. 2001; Ichise et al. 2003). To clarify whether in-vivo PET allows a side-by-side comparison of different agents acting on 5-HTT in the same experimental paradigm, as demonstrated in our previous ex-vivo occupancy study (Maeda et al. 2006b), and whether quantitative indices in rats can retrospectively predict the pharmacodynamics of a 5-HTT inhibitor in humans as assessed in our clinical PET analysis using [11C]DASB (Takano et al. 2006), we also conducted [11C]DASB PET scans of rats pretreated with fluvoxamine, one of the commonly used selective serotonin reuptake inhibitors (SSRIs).

Materials and methods

Drugs and chemicals

WF-516 was synthesized at Mitsubishi Tanabe Pharma Co. (Japan). Fluvoxamine maleate (fluvoxamine) and fluoxetine hydrochloride (fluoxetine) were obtained from Sigma-Aldrich (USA). Hydroxypropylmethyl cellulose (HPMC) was purchased from Shin-Etsu Chemical (Japan). All other chemicals were analytical grade and commercially available.

Animals

The research protocols of the present study were approved by the Animal Ethics Committee of the
National Institute of Radiological Sciences (NIRS). Twenty male Wistar rats aged 8 wk were purchased from Japan SLC (Japan) and kept in animal rooms maintained at 24 °C on a 12-h light/dark cycle (lights on 07:00 hours), with food and water available ad libitum. The mean body weight of the rats was 285 ± 70 (s.e.) g, ranging from 150 to 500 g. Prior to the PET measurements, neuroanatomical template images of the rat brain were generated by a high-resolution magnetic resonance imaging (MRI) system with a 400-mm bore, 7-T horizontal magnet (NIRS/KOBELCO, Japan; Bruker BioSpin, Germany) equipped with 120-mm diameter gradients (Bruker BioSpin), as described elsewhere (Maeda et al. 2007). A 72-mm diameter coil was used for radio frequency transmission, and signals were collected by a 4-channel surface coil. Coronal T2-weighted MR images were obtained by a fast spin-echo sequence with the following imaging parameters: repetition time = 400 ms, effective echo time = 48 ms, field of view (FOV) = 30 mm × 22.5 mm, nominal resolution = 117 µm × 117 µm, slice thickness = 600 µm and number of averages = 32.

Radioligand synthesis

[11C]DASB was synthesized by methylation of the corresponding des-methyl precursor with [11C]CH₃I (Wilson et al. 2000). Briefly, [11C]CH₃I, produced from 14CO₂, was swept by a flow of N₂ gas into a solution of des-methyl precursor in dimethylformamide at −15 °C. When radioactivity had peaked the solution was heated to 90 °C for 4 min and quenched with HPLC mobile phase. The mixture was purified by HPLC. The desired fraction ([11C]DASB was collected and evaporated to dryness at 50 °C and the residue taken up in 10 ml sterile saline. The saline solution of [11C]DASB was passed through a sterile 0.22-µm filter into a sterile, pyrogen-free bottle containing 400 µl of 15% ascorbic acid injection. An aliquot of the formulated solution was used to establish the chemical and radiochemical purity and specific activity of the final solution by analytical HPLC. Radiochemical purities were >95%, and specific radioactivity at the end of radiosynthesis was 269 ± 144 GBq/µmol.

Measurement of 5-HTT occupancy by drugs using PET

A series of four dynamic PET scans were performed for each rat ~5 h and 1 h after oral pretreatment with graded doses of Wi-516 (vehicle only, 0.1, 1 and 10 mg/kg) and fluvoxamine (vehicle only, 3, 10 and 30 mg/kg), respectively, dissolved or suspended in 0.5% HPMC. The scans for the same individual rats receiving Wi-516 (n = 4) and fluvoxamine (n = 7) were conducted more than 2 wk and 5 d apart, respectively. A complete recovery of [11C]DASB binding to the baseline level after these periods for washout of Wi-516 and fluvoxamine was confirmed by our initial pilot study consisting of two longitudinal scans along the time-course following the drug administration.

All PET scans were carried out using a microPET Focus 220 scanner (Siemens Medical Solutions, USA) designed for rodents and other small laboratory animals, which provides 95 transaxial slices 0.815 mm (centre-to-centre) apart, a 19.0-cm transaxial FOV, a 7.6-cm axial FOV, 1.3-mm in-plane resolution and 3.4% of absolute sensitivity at the centre of FOV for an energy window at 250–750 keV (Tai et al. 2005). The rats were anaesthetized with 1.5–2% isoflurane in air (21/min flow rate) ~30 min before measurements. Following transmission scans for attenuation correction using a 68Ge-68Ga point source, emission scans were acquired for 90 min in 3D list mode with an energy window of 350–750 keV, immediately after intravenous injection of [11C]DASB (at a dose of 104 ± 13 MBq and specific radioactivity of 191 ± 106 GBq/µmol at the time of injection). The injected mass of DASB ranged from 0.216 to 2.8 nmol, and averaged at 0.672 ± 0.408 (s.d.) nmol. In the fluvoxamine study, ~250 µl of blood samples were collected from the tail vein upon initiation of the scan. The collected blood was centrifuged, and plasma was frozen at −80 °C pending assays. The rats were kept warm by using a small-animal warmer/thermometer system (BWT-100; Bio Research Center, Japan). All list-mode data were stored into 3D sinograms, which were then Fourier-rebinned into 2D sinograms (26 frames: 4 × 1, 8 × 2, and 14 × 5 min). Images were reconstructed using 2D-filtered back-projection with a 0.5-mm Hanning filter. Regions of interest (ROIs) were placed on the striatum, thalamus, midbrain and cerebellum using PMOD® image analysis software (PMOD Group, Switzerland) with reference to the MRI template. Since male Wistar rats aged 8 wk are known to exhibit a rapid increase of the body weight in contrast to a subtle growth of the brain, their relative brain weight (% of body weight) decreases as a function of age. In light of this uneven growth of organs during a considerably long observation period (~8 and 4 wk in the Wi-516 and fluvoxamine treatment groups, respectively), we calculated the uptake of [11C]DASB as percentage of injected dose per tissue volume (% dose/ml) without corrections for the body weight of the animals, and determined the 5-HTT occupancy by using a reference tissue model, instead of an area-under-the-curve-based estimation. The
cerebellum was used as the reference tissue because of its negligible density of 5-HTT (Lin et al. 2004). Specific binding of $[^{11}C]$DASB was determined by subtraction of radioactivity in the cerebellum from that in each target ROI. Binding potential based on the specific binding compared to the non-displaceable uptake (BP<sub>ND</sub>) for $[^{11}C]$DASB was quantified by multilinear reference tissue model (MRTM) (Ichise et al. 2003). MRTM was initially used for estimating the rate constant, termed $k_2'$, for the efflux of the tracer from brain to plasma in the cerebellum. The $k_2'$ value was determined by averaging those calculated from the striatal, thalamic and midbrain data combined with cerebellar data, and BP<sub>ND</sub> in each of these ROIs was then estimated by redoing MRTM with fixed $k_2'$. The 5-HTT occupancy by Wf-516 and fluvoxamine was calculated using the following equation:

$$\text{Occ} = \frac{(\text{BP}_{ND, \text{vehicle}} - \text{BP}_{ND, \text{drug}}) \times 100}{\text{BP}_{vehicle}}$$

where Occ = occupancy, BP<sub>ND, vehicle</sub> and BP<sub>ND, drug</sub> are the BP<sub>ND</sub> values in PET analyses after pretreatment with vehicle only and test drug, respectively. Dose (ED<sub>50</sub>) and plasma concentration (EC<sub>50</sub>) of the drugs required for 50% occupancy of 5-HTT were determined according to the following relationships:

$$\text{Occ} = 100 \times C / (C + \text{EC}_{50}) \quad \text{and} \quad \text{Occ} = 100 \times D / (D + \text{ED}_{50})$$

where C and D are the plasma concentration and dose of the drugs in each experimental condition, respectively.

**Pharmacokinetics of fluvoxamine during isoflurane anaesthesia**

To examine the effects of isoflurane anaesthesia used for PET studies on the metabolism of fluvoxamine, plasma concentrations of fluvoxamine in rats under anaesthetized and unanaesthetized conditions were compared. Briefly, fluvoxamine at a dosage of 10 mg/kg was orally administered to nine rats not used for the PET studies, and ~250 μl of blood was sampled from the tail vein by incising the tail with a razor at 30 min of the treatment. Five of these rats were subsequently anaesthetized with isoflurane as described above, while the other four animals were left awake. Collection of blood was then done at 60 and 120 min of administration, and plasma samples obtained by centrifugation were stored at −80 °C until required.

**Measurement of plasma fluvoxamine concentration**

One hundred μl of plasma sample was combined with 10 μl of internal standard solution (200 ng/ml fluoxetine), 100 μl of methanol and 890 μl of 2% formic acid. The mixture was applied to a solid-phase extraction cartridge (Oasis<sup>®</sup> MCX; Waters, USA), which was then washed with 2% formic acid and methanol. Fluvoxamine in the solution was eluted with 1 ml of 5% ammonium hydroxide in methanol. The eluate was evaporated to dryness under a nitrogen stream, and the residue was reconstituted in 100 μl of equivalent mixture of HPLC mobile phase A composed of water/methanol/formic acid (90/10/0.05, v/v/v) and mobile phase B composed of water/methanol/formic acid (10/90/0.05, v/v/v), and filtrated with Millex-LH (Millipore, USA). Subsequently, 20 μl of the filtrate was injected into the LC/MS/MS system equipped with a high-performance liquid chromatograph (HP-1100; Agilent, USA) and a tandem mass spectrometer (TSQ-7000, Thermo Fisher Scientific, USA). HPLC analysis was performed on a CAPCELL PAK C18 column (UG120, 5 μm, 2.0 × 35 mm; Shiseido, Japan) at 40 °C. Mobile phase A/B (50/50, v/v) at a flow rate of 0.15 ml/min was used for the elution process, and the column was washed by raising the ratio of mobile phase B up to 100%. The eluted fluvoxamine and fluoxetine were ionized using the electrospray interface and detected by selected reaction monitoring of the transitions of m/z 319.5 to 70.8 and 310.7 to 44.4, respectively.

**Results**

Representative PET images of the same rat brains generated by summation of the dynamic data at 30–90 min after injection of $[^{11}C]$DASB at baseline and following oral pretreatment with Wf-516 and fluvoxamine are shown in Fig. 1. Consistent with the spatial distribution of 5-HTT (D’Amato et al. 1987), injection of $[^{11}C]$DASB into rats without pretreatment gave rise to intense radioactive signals primarily in the ventral striatum, thalamus, hypothalamus, amygdala and midbrain. Accumulation of the radioligand at modest levels was also observed in the dorsal striatum, thalamus, hypothalamus, amygdala and midbrain. Low but significant levels of radioactivity were noted in the neocortex and pons, and the cerebellum exhibited the lowest level of tracer retention. A comparison of the changes in the radioligand signal intensities with increments of Wf-516 and fluvoxamine dosages was performed using the ratio of BP<sub>ND</sub> and the dosage ranges for these drugs apparently included ED<sub>50</sub>.
The dose-dependent reduction of \([^{11}C]\)DASB binding to 5-HTT in the pretreatment studies was also demonstrated by alterations of time–radioactivity curves in the striatal, thalamic and midbrain ROIs (Fig. 2). The curves were responsive to small dosages of Wf-516 (0.1 mg/kg) and fluvoxamine (3 mg/kg). In line with the illustration, 10 mg/kg Wf-516 and 30 mg/kg fluvoxamine lowered the retention of \([^{11}C]\)DASB at 30–60 min of the scans to a level close to that in the cerebellum. Specific binding of the radioligand to 5-HTT was then estimated as the difference in radioactivity between target and cerebellar ROIs, and was plotted against time (Fig. 3). The binding peaked at 15–20 min of the PET scans at baseline and after low- and moderate-dose pretreatments, while the peaks were achieved much earlier (<5 min) in the studies with the highest dosage of each drug, implying that the curves reflect not only specific radiotracer binding but also the difference in tracer delivery between target and reference regions, particularly at a relatively early phase of the measurement. As the transient peak equilibrium was also reached within the first quarter of the total imaging time, this observation did not justify a simple estimation of the radiotracer binding based on the maximum values in the plots as an adequate method for determining the occupancy of 5-HTT by drugs.

Attenuation of the specific binding of \([^{11}C]\)DASB exhibited a dependency on the dosages of Wf-516 and fluvoxamine (Table 1). There was no significant difference in 5-HTT occupancy by Wf-516 at any dosage among the three target regions examined here \((p > 0.05\) by two-way repeated-measures ANOVA), while that by fluvoxamine was influenced by a significant interaction between dosage and region \([p < 0.05, F(4, 24) = 5.12\) by two-way repeated-measures ANOVA], primarily due to inter-regional variability of the occupancy at 3 mg/kg. According to the temporal profiles of specific radioligand binding (Fig. 3b), this variability can be attributed to the regionally different pattern of changes in the initial portion of the curve by 3 mg/kg fluvoxamine relative to the baseline, which was not found in the data for Wf-516 (Fig. 3) and may be largely influenced by alterations in radiotracer delivery. Hence, we assumed that the occupancy of 5-HTT by Wf-516 and fluvoxamine exhibited no or only minimal divergence among the three regions examined here, and used the following mean occupancy values of these regions as representative indices: 1.7±1.5%, 28.2±4.2% and 72.3±1.2% at 0.1, 1 and 10 mg/kg Wf-516, respectively (mean±S.E. in four rats), and 4.2±3.9%, 29.3±2.6% and 84.9±2.4% at 3, 10 and 30 mg/kg fluvoxamine, respectively (mean±S.E. in seven rats).

Scatterplots of the mean 5-HTT occupancies against the doses permitted a comparison of Wf-516 and fluvoxamine in terms of their in-vivo binding to central 5-HTT (Fig. 4). The ED\(_{50}\) estimates for Wf-516 and fluvoxamine were 3.1 mg/kg [95% confidence interval (CI) 2.4–3.9] and 15.2 mg/kg [95% CI 9.7–20.8], respectively. These values implied that relative binding potencies of the two agents were fairly consistent with our previous ex-vivo data (Wf-516 vs. fluvoxamine: 1.1 mg/kg vs. 4.5 mg/kg) (Maeda et al. 2006b), as there...
was only a 3-fold difference between these assays. The curve fit of the fluvoxamine study \( (r^2 = 0.7848) \) was slightly worse than that of the Wf-516 study \( (r^2 = 0.9625) \), primarily due to relatively large inter-subject variability of the occupancy values at each dosage. The fit was notably improved by plotting the occupancy against the plasma fluvoxamine concentration \( (r^2 = 0.9046) \), as illustrated in Fig. 5, indicating considerable divergence in the time-course of fluvoxamine elimination from plasma among individuals. The EC\textsubscript{50} value determined from this plot was 6.1 ng/ml (95% CI 3.6–8.6), and was in good agreement with the value (4.6 ng/ml) reported in our previous PET study for healthy human subjects (Takano et al. 2006), in which the same radioligand and analytical model as those in the present work were employed.

Fig. 2. Time–radioactivity curves for \(^{11}C\)DASB after pretreatments with (a) Wf-516 and (b) fluvoxamine at different dosages. Radiotracer uptake into each region is expressed as percentage of injected dose per tissue volume (% dose/ml). Bars indicate s.e. \( (n = 4 \) and 7 in Wf-516 and fluvoxamine studies, respectively).
The temporal profile of the plasma fluvoxamine level (Table 2) was not significantly influenced by anaesthesia with isoflurane during the observation period of 120 min ($p > 0.1$ for the main effect of anaesthesia and interaction between time and anaesthesia, by two-way repeated-measures ANOVA), although there was a tendency for accelerated elimination of fluvoxamine from plasma by the use of anaesthetic.

**Discussion**

The present $[^{14}]$C]DASB-PET data provide the first demonstration, to our knowledge, of the consistency of EC$_{50}$ values for a drug acting on a neurotransmission component between preclinical and clinical (Takano et al. 2006) PET studies using the same radioligand.

Taken together with the pharmacokinetic properties of the tested drug (i.e. fluvoxamine), this provides insights into the pharmacokinetic requirements for a drug to be used for a preclinical PET assay for predicting plasma–brain relationships in humans. For this purpose, it is also of methodological significance to collect blood samples during the PET experiments and determine concentrations of the drug in plasma. The extension of this view highlights the usefulness of in-vivo 5-HTT imaging in rat brains for preclinically testing new candidate drugs, exemplified by Wf-516, on the basis of molecular pharmacodynamics. The small-animal PET system applied here has also been demonstrated to provide a quantitative index for the magnitude of pharmacological intervention in central serotonergic neurotransmission, which will further

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**Fig. 3.** Time-course of specific $[^{14}]$C]DASB binding to 5-HTT in the striatum (top panels), thalamus (middle panels) and midbrain (bottom panels) after pretreatments with (a) Wf-516 and (b) fluvoxamine at different dosages. Binding was estimated as the difference in radiotrace signals between the target and cerebellar regions, and expressed as percentage of injected dose per tissue volume (% dose/ml). Bars indicate S.E. ($n = 4$ and 7 in Wf-516 and fluvoxamine studies, respectively).
permit neurochemical assessments relevant to behavioural phenotypes in the treatment of normal animals and disease models.

It is of particular significance that there was a correlation between the plasma concentration of fluvoxamine and the occupancy of 5-HTT by this compound (Fig. 5), as this finding justifies the use of plasma measures as surrogates for the effects of fluvoxamine on central 5-HTT in future analyses of animals with a larger sample size. These data, again, are in agreement with the results in our clinical PET study (Takano et al. 2006), implying the inter-species consistency of kinetic relationships between concentrations of fluvoxamine in the plasma and brain tissue compartments, notwithstanding the considerable discrepancy of metabolic and clearance rates for this compound between rats and humans (Sato et al. 1995; van Harten, 1995).

High similarities of EC\textsubscript{50} values and plasma-occupancy curves for a drug among species are achievable if its metabolism generates no pharmaco-logically active by-products, as in the catabolic process of fluvoxamine (Wilde et al. 1993), or if the production of active metabolites occurs at an equivalent rate in these species. Additionally, a previous in-vitro assay showed that the proportion of fluvoxamine (100 ng/ml) bound to proteins in rat serum (85.5\%) was fairly close to that in human serum (79.0\%) (Sato et al. 1995), supplementing evidence for the translatability of fluvoxamine data between preclinical and clinical investigations. Although determination of ED\textsubscript{50} values was previously performed in a well-designed PET measurement of 5-HT\textsubscript{1A} receptors in rats pretreated with pindolol (Hirani et al. 2000), assessments of relationships between the plasma level and specific

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Region</th>
<th>Wf-516</th>
<th>Fluvoxamine</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Vehicle 0.1 mg/kg</td>
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<tr>
<td>BP\textsubscript{ND}</td>
<td>Striatum</td>
<td>3.05 ± 0.30</td>
<td>3.02 ± 0.27</td>
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<td></td>
<td>Thalamus</td>
<td>3.01 ± 0.20</td>
<td>2.88 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>Midbrain</td>
<td>2.40 ± 0.12</td>
<td>2.39 ± 0.10</td>
</tr>
<tr>
<td>Occupancy (%)</td>
<td>Striatum</td>
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<td>0.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Thalamus</td>
<td>–</td>
<td>4.7 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>Midbrain</td>
<td>–</td>
<td>-0.3 ± 7.4</td>
</tr>
<tr>
<td>Mean of 3 regions</td>
<td>–</td>
<td>1.7 ± 1.5</td>
<td>28.2 ± 4.2</td>
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</tbody>
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BP\textsubscript{ND}: Binding potential based on specific binding compared to non-displaceable uptake. Data represent mean ± s.e. of four rats for Wf-516 and seven rats for fluvoxamine.

Fig. 4. Relationship between oral dose of pretreatment drugs and 5-HTT occupancy. Mean values of occupancies in the striatum, thalamus, and midbrain are plotted against the dose of (a) Wf-516 and (b) fluvoxamine. Open circles represent individual values. Regression curves were generated by the following equation: Occ = 100 \times D/(D + ED\textsubscript{50}), where Occ is 5-HTT occupancy and D is the dose of the drugs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Region</th>
<th>Vehicle</th>
<th>0.1 mg/kg</th>
<th>1 mg/kg</th>
<th>10 mg/kg</th>
<th>Vehicle</th>
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<td>3.02 ± 0.27</td>
<td>2.31 ± 0.11</td>
<td>0.99 ± 0.07</td>
<td>3.17 ± 0.15</td>
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<td>2.28 ± 0.11</td>
<td>0.52 ± 0.08</td>
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<td></td>
<td>Thalamus</td>
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<td>2.14 ± 0.20</td>
<td>0.82 ± 0.08</td>
<td>3.15 ± 0.15</td>
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<tr>
<td></td>
<td>Midbrain</td>
<td>2.40 ± 0.12</td>
<td>2.39 ± 0.10</td>
<td>1.62 ± 0.19</td>
<td>0.55 ± 0.06</td>
<td>2.50 ± 0.09</td>
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<td>1.80 ± 0.11</td>
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<tr>
<td>Occupancy (%)</td>
<td>Striatum</td>
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<td>0.8 ± 0.1</td>
<td>23.0 ± 6.1</td>
<td>67.0 ± 2.4</td>
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<td>–</td>
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<td>Thalamus</td>
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<td>72.7 ± 1.6</td>
<td>–</td>
<td>–</td>
<td>5.1 ± 3.0</td>
<td>32.1 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>Midbrain</td>
<td>–</td>
<td>-0.3 ± 7.4</td>
<td>32.4 ± 8.7</td>
<td>77.0 ± 2.4</td>
<td>–</td>
<td>–</td>
<td>9.6 ± 5.2</td>
<td>28.0 ± 4.0</td>
</tr>
<tr>
<td>Mean of 3 regions</td>
<td>–</td>
<td>1.7 ± 1.5</td>
<td>28.2 ± 4.2</td>
<td>72.3 ± 1.2</td>
<td>–</td>
<td>–</td>
<td>4.2 ± 3.9</td>
<td>29.3 ± 2.6</td>
<td>84.9 ± 2.4</td>
</tr>
</tbody>
</table>

Table 1. BF\textsubscript{ND} of [\textsuperscript{11}C]DASB and occupancy of 5-HTT by Wf-516 and fluvoxamine.
binding in the brain for a drug may be required for linking small-animal PET data to human studies. There has been only one report on the use of PET for examining such relationships in rats. Attack and colleagues documented that EC$_{50}$ of lorazepam for occupancy of central benzodiazepine receptors was ~100 ng/ml based on [11C]flumazenil PET data (Attack et al. 2007), which corresponds to the dose inducing only 6–9% occupancy in human PET measurement with the same tracer (Lingford-Hughes et al. 2005). It should be noted that regional densities of flumazenil-binding sites may differ among species, as suggested by a previous autoradiographic assay using a related agonistic ligand for $\gamma$-aminobutyric acid type A receptor (Duncan et al. 1998). Moreover, while intralogic processing of lorazepam does not yield neuroactive metabolites, the dissociation of EC$_{50}$ measures between rats and humans is attributable to the high percentage of plasma lorazepam bound to proteins (~90%) (Johnson et al. 1979). Due to this property, a subtle difference in the fraction of protein-bound lorazepam in plasma among species may result in a great variability of EC$_{50}$ values, as the unbound concentration of this compound is associated with the uptake into the brain (Arendt et al. 1987). Assessments based on such a rationale would also be conducted for Wf-516 by measuring its plasma concentration, after fully investigating its systemic kinetics including identification of the presence or absence of its active metabolites, and the resultant information would bring prospective insights into the effective dosage of this new drug in humans.

The ED$_{50}$ values for Wf-516 and fluvoxamine (Fig. 4) indicate the 5-HTT-binding activity of Wf-516 relative to the representative SSRIs. Furthermore, our previous behavioural study indicated that ED$_{50}$ values for the antagonism by Wf-516 and fluvoxamine to $p$-chloroamphetamine-induced hyperlocomotion in rats were 1.7 mg/kg and 14 mg/kg, respectively (Maeda et al. 2006b), suggesting that the PET index is considerably equivalent to not only ex-vivo binding but also in-vivo behavioural potencies. In addition, our previous examinations have revealed that Wf-516 also acts on 5-HT$_{1A}$ receptors (El Mansari & Blier, 2008; Maeda et al. 2006a,b), reinforcing the notion of the potency of this drug in augmenting central serotonergic neurotransmission.

The matching of in-vivo and ex-vivo data on the occupancy of 5-HTT also suggests that the mass effects inevitable in small-animal PET imaging do not compromise the accuracy of pharmacokinetic analysis using the present system and radioligand. By applying the radioactive dose (~600 MBq) and specific radioactivity (~240 GBq/μmol) of [11C]DASB in our clinical PET scans (Takano et al. 2006), body weight of humans (~60 kg), and the reported ED$_{50}$ of this tracer (56 nmol/kg) (Wilson et al. 2002) to the equation proposed in a previous study (Hume et al. 1998), the $^{11}$C-labelled plus cold DASB in the human PET assay is estimated to occupy only 0.07% of central 5-HTT, and is accordingly unlikely to fluctuate the results of kinetic analysis to a noticeable extent. Similarly, the occupancy of rat 5-HTT by the same tracer is calculated as 3.2% by referring to the parameters in this study (radioactive dose, 104 MBq; specific radioactivity, 191 GBq/μmol; body weight, 0.3 kg). Since the threshold of the occupancy by an imaging agent below which conventional kinetic models are reasonably applicable is conceived as 1% (Hume et al. 1998) or 5% (Jagoda et al. 2004), quantification of available 5-HTT by $^{11}$C]DASB PET is possible but needs validation, as demonstrated here. In considering this, a key issue for

### Table 2. Plasma concentration of fluvoxamine in awake and isoflurane-anaesthetized rats

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Awake (ng/ml)</th>
<th>Isoflurane (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>13.1 ± 6.3</td>
<td>10.4 ± 4.5</td>
</tr>
<tr>
<td>60</td>
<td>12.1 ± 4.5</td>
<td>4.0 ± 1.1</td>
</tr>
<tr>
<td>120</td>
<td>5.1 ± 1.8</td>
<td>2.7 ± 0.6</td>
</tr>
</tbody>
</table>

Data represent mean ± s.e.
reducing this occupancy to a level below the threshold in rodents may be the specific radioactivity of the ligand, because additional decreases of its injected dose might not be readily allowed, based on the sensitivity of the current small-animal PET scanner (absolute sensitivity of 3.4% at the centre of FOV) (Tai et al. 2005). In fact, the specific radioactivity of [14C]DASB synthesized here was much higher than previous rat studies, which may account for the larger BPND values in our measurements (e.g. striatal BPND estimated as 3.1–3.2 and 0.8–0.9, respectively, in the present study and a previous report by Lundquist et al. (2007). Additionally, accuracies of the occupancy determination could be influenced by the specific radioactivity, particularly in quantifying a small amount of available binding sites after the blockade of a large proportion of 5-HTT by the pre-administered drug. This is of notable significance in the application of [14C]DASB, since the level of its non-specific binding was shown to be high (Ginovart et al. 2001) compared to commonly used neuroreceptor ligands such as [14C]raclopride (Lammertsma et al. 1996). The robustness of the occupancy assay using [14C]DASB with high specific radioactivity might be supported by the consistency of EC50 for fluvoxamine between rats and humans, despite a considerable difference in BPND between the present rat and previous human (Takano et al. 2006) and cat (Ginovart et al. 2003) PET studies with [14C]DASB. We also postulate that further experiments, that vary these parameters and test other radioligands with different affinities for 5-HTT, would contribute to a more stringent definition of the above-mentioned threshold in order to optimize the measurement condition for drug occupancy studies.

Besides the specific radioactivity, the collection of individual data on the plasma concentration of fluvoxamine is essentially important for the accurate determination of EC50, as the same dosage of this drug caused a large variability of its plasma concentration. The influence of isoflurane anaesthesia on the plasma fluvoxamine level was insignificant in our pharmaco-kinetic assessments, and therefore this variability is attributable to the diversity of the bioavailability. In fact, the bioavailability of fluvoxamine is only ~50% notwithstanding its excellent absorption from the gastrointestinal tract, primarily due to a strong first-pass effect (Sato et al. 1995). Hence, the plasma fluvoxamine concentration is largely dependent on the individual status of the first-pass metabolism. Moreover, oral administration of fluvoxamine exceeding the maximum capacity of the first-pass metabolism may give rise to a discontinuous increase of its bioavailability, leading to an additional variability of the plasma level. Although isoflurane did not overtly alter the plasma kinetics of fluvoxamine, it remains to be elucidated whether the uptake of [14C]DASB can be affected by anaesthetics. A recent report on 3F-labelled ligands for 5-HTT documented no prominent influences of anaesthetics in monkeys compared to an awake condition (Stehouwer et al. 2008), and a similar experimental paradigm could be applied to rat PET analyses.

Unlike in-vitro and ex-vivo assays of excised brains, PET measurements allow intra-subject comparisons of neurotransmission parameters in different therapeutic conditions, which also strengthen statistical power and efficient data acquisition with reasonably high throughput. In utilizing these advantages, preclinical PET study offers significant insights into the mechanistic basis of psychobehavioural characteristics observed in small animals modelling mental illnesses and their therapies, provided the experimental settings that enable reliable preclinical evaluation are sufficiently clarified.

Statement of Interest

None.

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References


