Fenfluramine Reduces $[^{11}\text{C}]$Cimbi-36 Binding to the 5-HT$_{2A}$ Receptor in the Nonhuman Primate Brain

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Abstract

Background: $[^{11}\text{C}]$Cimbi-36 is a serotonin 2A receptor agonist positron emission tomography radioligand that has recently been examined in humans. The binding of agonist radioligand is expected to be more sensitive to endogenous neurotransmitter concentrations than antagonist radioligands. In the current study, we compared the effect of serotonin releaser fenfluramine on the binding of $[^{11}\text{C}]$Cimbi-36, $[^{11}\text{C}]$MDL 100907 (a serotonin 2A receptor antagonist radioligand), and $[^{11}\text{C}]$AZ10419369 (a serotonin 1B receptor partial agonist radioligand with established serotonin sensitivity) in the monkey brain.

Methods: Eighteen positron emission tomography measurements, 6 for each radioligand, were performed in 3 rhesus monkeys before or after administration of 5.0 mg/kg fenfluramine. Binding potential values were determined with the simplified reference tissue model using cerebellum as the reference region.

Results: Fenfluramine significantly decreased $[^{11}\text{C}]$Cimbi-36 (26–62%) and $[^{11}\text{C}]$AZ10419369 (35–58%) binding potential values in most regions ($p < 0.05$). Fenfluramine-induced decreases in $[^{11}\text{C}]$MDL 100907 binding potential were 8% to 30% and statistically significant in 3 regions. Decreases in $[^{11}\text{C}]$Cimbi-36 binding potential were larger than for $[^{11}\text{C}]$AZ10419369 in neocortical and limbic regions (~35%) but smaller in striatum and thalamus (~40%). Decreases in $[^{11}\text{C}]$Cimbi-36 binding potential were 0.9 to 2.8 times larger than for $[^{11}\text{C}]$MDL 100907, and the fraction of serotonin 2A receptor in the high-affinity state was estimated as 54% in the neocortex.

Conclusions: The serotonin sensitivity of serotonin 2A receptor agonist radioligand $[^{11}\text{C}]$Cimbi-36 was higher than for antagonist radioligand $[^{11}\text{C}]$MDL 100907. The serotonin sensitivity of $[^{11}\text{C}]$Cimbi-36 was similar to $[^{11}\text{C}]$AZ10419369, which is one of the most sensitive radioligands. $[^{11}\text{C}]$Cimbi-36 is a promising radioligand to examine serotonin release in the primate brain.

Keywords: $[^{11}\text{C}]$Cimbi-36, $[^{11}\text{C}]$AZ10419369, $[^{11}\text{C}]$MDL 100907, fenfluramine, serotonin
Introduction
The serotonin (5-HT) neurotransmission system has an important role in the regulation of basic physiological functions as well as in higher brain functions such as emotion and cognition (Cools et al., 2011; Chou et al., 2012). It is of great interest to develop noninvasive methods to examine the serotonin system in the living primate brain, since a role of this system is implicated in the pathophysiology and treatment of major psychiatric disorders. By consequence, several radioligands have long since been developed for positron emission tomography (PET) imaging of serotonin receptor subtypes and the serotonin transporter (Saulin et al., 2012; Paterson et al., 2013). More recently, it has been of particular interest to develop radioligands that are sensitive to changes in the endogenous neurotransmitter concentration (Laruelle, 2000; Finnema et al., 2015). This PET imaging paradigm is typically interpreted according to the competition model, which postulates that binding of radioligand to neurotransmitter will decrease after increases in neurotransmitter concentration, and vice versa. For that purpose, there is need for radioligands that are sensitive to the endogenous 5-HT concentration in brain.

Some PET radioligands for the 5-HT₁₃ receptor and the 5-HT₂₃ receptor such as [¹¹C]CUMI-101, [¹¹C]AZ10419369, and [¹¹C]P943 have indeed been reported to be sensitive to drug-induced changes in the 5-HT concentration (Finnema et al., 2015). We have previously demonstrated that [¹¹C]AZ10419369, a 5-HT₁₃ receptor partial agonist radioligand, is sensitive to increases in serotonin concentration, as fenfluramine reduced [¹¹C]AZ10419369 binding up to 50% in the monkey brain (Finnema et al., 2010b, 2012). Furthermore, [¹¹C]AZ10419369 allowed detection of the relatively low changes in 5-HT concentration in the human brain induced by escitalopram, a selective serotonin reuptake inhibitor (Nord et al., 2013). As modulation of multiple target proteins is one of the current strategies in the development of novel psychotrophic drugs (Bang-Andersen et al., 2011; Varnäs et al., 2016), it is important to develop 5-HT-sensitive PET radioligands targeting 5-HT receptor subtypes other than 5-HT₁₃ and 5-HT₂₃. It has been suggested that the 5-HT₁₃ receptor could be a promising target for that purpose (Paterson et al., 2010; Tyacke and Nutt, 2015). However, in initial PET studies in the primate brain using 5-HT₁₃ receptor antagonist radioligands such as [¹¹C]MDL100907, no or limited 5-HT sensitivity has been reported (Paterson et al., 2010; Quednow et al., 2012; Talbot et al., 2012; Finnema et al., 2015).

The binding of agonist radioligands has been proposed to be more sensitive to alterations in neurotransmitter concentration than the binding of antagonist radioligands at the same target protein (Paterson et al., 2010; Finnema et al., 2015). This hypothesis has been based on the ternary complex model that suggests that receptors can be in 2 functional states (De Lean et al., 1980; Finnema et al., 2010a). Whereas agonists have high affinity to the functional G-protein coupled receptor and low affinity to the G-protein uncoupled receptor, antagonists have similar affinity to both states of the receptor. Therefore, neurotransmitters (being endogenous agonists) may more markedly compete with radioligand binding to the high-affinity state receptor than to the low-affinity state receptor. This hypothesis has been supported by the higher dopamine sensitivity of dopamine D₂/D₃ receptor agonist radioligands than antagonists (Narendran et al., 2004, 2010; Seneca et al., 2006; Shotbolt et al., 2012; Gallezot et al., 2014). Considering the low fraction (13%-45%) of 5-HT₂₃ receptors in the high-affinity state in vitro (Sleight et al., 1996; Fitzgerald et al., 1999; Gray et al., 2003; Hazelwood and Sanders-Bush, 2004), there may be prominent differences in 5-HT sensitivity between agonist and antagonist radioligands for this receptor subtype.

[¹¹C]Cimbi-36 is the first 5-HT₂₃ receptor agonist radioligand (Ettrup et al., 2011) that has been characterized both in nonhuman primates (NHPs) (Finnema et al., 2014) and humans (Ettrup et al., 2014). The 5-HT sensitivity of [¹¹C]Cimbi-36 has recently been evaluated in the pig brain (Jørgensen et al., 2016), confirming that [¹¹C]Cimbi-36 binding was sensitive to the 4- to 11-fold increases in 5-HT concentration demonstrated by microdialysis. Therefore, [¹¹C]Cimbi-36 might be a promising radioligand for measurement of increases in 5-HT concentration in the living primate brain.

The aim of this study was to evaluate the 5-HT sensitivity of [¹¹C]Cimbi-36 in the NHP brain. Using a within-subject comparison study design, the sensitivity was directly compared with that of [¹¹C]MDL100907 binding to the 5-HT₁₃ receptor, a reference radioligand with established serotonin sensitivity, and that of the 5-HT₁₃ receptor antagonist radioligand [¹¹C]MDL100907 (Ito et al., 1998). Fenfluramine was the drug administered to induce 5-HT release (Rothman and Baumann, 2002). The study design also allowed for an estimation of the fraction 5-HT₁₃ receptors in the high affinity state (Narendran et al., 2004). We hypothesized that the 5-HT sensitivity of [¹¹C]Cimbi-36 would be higher than for [¹¹C]MDL100907 and different from [¹¹C]AZ10419369 due to differences in the affinity of 5-HT to the target receptors (Paterson et al., 2010).

Methods
Subjects
The NHP study was approved by the Animal Research Ethical Committee of the Northern Stockholm region (Dnr N386/09 and N452/11). Three female rhesus monkeys (Macaca mulatta) with a mean body weight of 7.9 kg (range: 5.2–13.5 kg) were included.
The caring and experimental procedures were performed according to the Guidelines for planning, conducting and documenting experimental research (Dnr 4820/06-600) of Karolinska Institutet and the Guide for the Care and Use of Laboratory Animals: Eighth Edition (Council, 2011).

Preparation of Radioligands

$[^{11}C]Cimbi-36$, $[^{11}C]MDL$ 100907, and $[^{11}C]AZ10419369$ were prepared according to procedures reported previously (Lundkvist et al., 1996; Pierson et al., 2008; Andersson et al., 2011; Ettrup et al., 2011).

Study Design

A total of 18 PET measurements (6 for each radioligand) were performed on 9 experimental days. On each experimental day, a baseline PET measurement was performed in the morning and repeated after pretreatment with fenfluramine 5 mg/kg. The 2 PET measurements were performed approximately 3 hours apart. The fenfluramine dose was selected to achieve a substantial increase in 5-HT concentration and has been demonstrated to induce a 20-fold increase in a microdialysis study in monkeys (Udo de Haes et al., 2006). Racemic fenfluramine was formulated in saline and was i.v. infused (1 mL/kg) over 10 minutes, starting 30 minutes before injection of radioligand.

PET Experimental Procedures

Anesthesia was initiated by intramuscular injection of ketamine (~10 mg/kg) and, after tracheal intubation maintained by a mixture of sevoflurane (2%-8%), oxygen, and medical air. PET measurements were conducted in High Resolution Research Tomograph. A 6-minute transmission scan (using a single $^{133}$Cs source) was followed by acquisition of list-mode data for 123 minutes after i.v. bolus injection of radioligand. During the PET measurements after fenfluramine pretreatment, 7 venous blood samples (at -40, -5, 15, 30, 60, 90, and 120 minutes after injection of radioligand) were collected for determination of the fenfluramine concentration in plasma.

Determination of Plasma Fenfluramine and Norfenfluramine Concentrations

The main metabolite of fenfluramine, norfenfluramine, is also a potent 5-HT releaser (Rothman and Baumann, 2002). Therefore, the plasma concentrations of both fenfluramine and norfenfluramine were determined using liquid chromatography-mass spectrometry as described in supplementary Materials and Methods.

Magnetic Resonance Imaging (MRI)

T1-weighted MRI images were acquired for each monkey on a GE 1.5 Tesla Signa MRI scanner using a 3D spoiled gradient recalled protocol with repetition time 21 milliseconds, flip angle 35°, FOV 12.8, matrix $256 \times 256 \times 128$, and $128 \times 1.0$ mm$^2$ slices.

Image Data Analysis and Quantification

The MRI images were reoriented to the anterior-posterior commissure plane, and non-brain tissues were removed manually using the Image Processing and VOI Analysis Tool (PBAS) in FMOD (version 3.704; FMOD Technologies). The processed brain MRI images were then corrected for inhomogeneous intensity by applying the N4 algorithm (Tustison et al., 2010) using the Advanced Normalization Tools software package (http://stnava.github.io/ANTs/). PET images were preprocessed according to previously reported methods (Varrone et al., 2009) with reconstructed image frames binned as: 9 x 10 seconds, 2 x 15 seconds, 3 x 20 seconds, 4 x 30 seconds, 4 x 60 seconds, 4 x 180 seconds, and 17 x 360 seconds.

For each of the 9 baseline measurements, a summed PET image was generated for PET-MRI co-registration. Time frames for the summed PET image were based on high tissue counts and optimal tissue contrast to enable PET-MRI co-registration. The applied time frames were 12 to 63 minutes for $[^{11}C]Cimbi-36$, 21 to 75 minutes for $[^{11}C]MDL$ 100907, and 5 to 18 minutes for $[^{11}C]AZ10419369$. Each summation image was co-registered to its individual MRI brain image by the Rigid matching algorithm with default settings for primate in the FMod Fuse It Tool (PFUSEIT). The resulting transformation matrices were applied to the 2 PET data sets obtained for each monkey on the same day.

Fourteen volumes of interest (VOIs) were defined based on the NeuroMaps atlas in the INIA19 rhesus template (Rohlffing et al., 2012), including 3 striatal regions: putamen, caudate nucleus (CN), and ventral striatum (VS); 4 neocortical regions: frontal cortex (FC), parietal cortex (PC), temporal cortex (TC), and occipital cortex (OC); 3 limbic regions: anterior cingulated cortex (ACC), amygdala, and hippocampus; and finally, VOIs for thalamus, midbrain, cerebellum, and whole brain. The VOIs were selected based on regional 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor distribution and are similar as in previous PET studies in NHPs using $[^{11}C]Cimbi-36$, $[^{11}C]MDL$ 100907, and $[^{11}C]AZ10419369$ (Finnema et al., 2012, 2014). Each monkey’s brain MRI image was normalized to the INIA19 rhesus template by the Deformable matching algorithm with default settings for primate in the PFUSEIT, and the resulting normalization matrix was used to inversely transform the template VOIs into the individual MRI space.

Calculation of Binding Potential ($BP_{ND}$) and Change after Pretreatment

For each VOI, a decay-corrected time–activity curve was generated from the co-registered dynamic PET data. $BP_{ND}$ values were calculated using the simplified reference tissue model (Lammertsma and Hume, 1996), with cerebellum as the reference region (Meyer et al., 2010; Varnäis et al., 2011; Talbot et al., 2012; Finnema et al., 2014). The identifiability of $BP_{ND}$ values was evaluated by the estimate of SE during the fitting process by using the Marquardt-Levenberg algorithm (Marquardt, 1963) and expressed as the percentage of SE (%SE), calculated according to the following equation:

$$\%SE = \frac{\text{Standard error in } BP_{ND} \text{ estimation}}{BP_{ND} \text{ estimation}} \times 100. \quad (1)$$

The relative change in $BP_{ND}$ values ($\Delta BP_{ND}$) (%) was calculated using the following equation:

$$\Delta BP_{ND} (%) = \frac{(BP_{ND})_{\text{Pretreatment}} - (BP_{ND})_{\text{Baseline}}}{(BP_{ND})_{\text{Baseline}}} \times 100. \quad (2)$$

Statistical Analysis

Changes in parameters between the 2 PET measurements performed on the same day were assessed by paired t test. All statistical analyses were performed in GraphPad Prism (version...
Results

Radiochemistry

The mean radiochemical purity for each of 3 injected radioligands was 99% (range: 97–100%, n = 6 for each radioligand). Comparing radioligand injection parameters between baseline and pretreatment PET measurements for the same radioligand, a slightly higher injected radioactivity of [11C]AZ10419369 after pretreatment (188 MBq) than baseline (183 MBq) and higher injected mass of [11C]MDL 100907 after pretreatment (0.21 μg) than baseline (0.17 μg) were observed. Otherwise, there were no significant differences (supplementary Table 1).

Plasma Concentrations of Fenfluramine and Norfenfluramine

The time courses for the mean plasma concentrations of fenfluramine and norfenfluramine are presented in Figure 1. During the PET measurement, the mean plasma concentration values of fenfluramine and norfenfluramine were similar for the 9 experimental days. The mean (n = 3) values for fenfluramine and norfenfluramine were 2.39 μM and 0.75 μM after [11C]Cimbi-36, 2.44 μM and 0.86 μM after [11C]MDL 100907, and 2.59 μM and 0.83 μM after [11C]AZ10419369, respectively.

Fenfluramine-Induced Changes in Radioligand Binding

Regional BP
ND values could be reliably identified (%SE ≤ 10%) for most regions (Table 1), although the identifiability was slightly weaker for the VS (11.3 ± 2.3%, mean ± SD, n = 6), amygdala (10.5 ± 3.5%), and MB (16.5 ± 9.5%) in the [11C]Cimbi-36 experiments. Following administration of fenfluramine, radioactivity concentration in a majority of examined VOIs was reduced when compared with baseline (Figure 2). The reduction in [11C]Cimbi-36 BP
ND was statistically significant in 12 of the 13 examined brain regions (except putamen). For [11C]AZ10419369, BP
ND was also significantly reduced in all 13 brain regions. In contrast, the regional reduction in [11C]MDL 100907 BP
ND was not significant in most regions except for 3 (TC, ACC, and thalamus) (Table 1; Figure 3). For the whole brain, the mean reductions in BP
ND for [11C]MDL 100907, [11C]Cimbi-36, and [11C]AZ10419369 were 25.8%, 49.4%, and 46.3%, respectively.

Fenfluramine-induced [11C]Cimbi-36 ΔBP
ND was larger than [11C]MDL 100907 ΔBP
ND (Table 1). There were regional differences in fenfluramine-induced ΔBP
ND between [11C]Cimbi-36 and [11C]AZ10419369 as the decreases in [11C]Cimbi-36 BP
ND were larger than for [11C]AZ10419369 in neocortical and limbic regions (~35%), but smaller in striatum and thalamus (~40%) (Table 1).

Discussion

The aim of the present study in NHPs was to examine the sensitivity of the 5-HT
2C receptor agonist radioligand [11C]Cimbi-36 for changes in the endogenous serotonin concentration. A main observation was that the binding of [11C]Cimbi-36 was sensitive to 5-HT release induced by fenfluramine. The results are consistent with a recent PET study in pigs in which fenfluramine reduced the BP
ND values by 44% (Jørgensen et al., 2016). Moreover, the agonist radioligand [11C]Cimbi-36 was more sensitive to 5-HT release than the antagonist 5-HT
2A receptor radioligand [11C]MDL100907. In addition, the 5-HT sensitivity of [11C]Cimbi-36 binding was comparable with that for [11C]AZ10419369 binding to the 5-HT
2A receptor. In summary, [11C]Cimbi-36 appears to be one of the most sensitive radioligands so far developed for detection of changes in the endogenous 5-HT concentration in the primate brain.

The larger fenfluramine-induced change in ΔBP
ND for [11C]Cimbi-36 than for [11C]MDL 100907 is in line with the previously reported higher dopamine sensitivity of dopamine D/D
3 receptor agonist radioligands than antagonist radioligands such as [11C]raclopride (Narendran et al., 2004, 2010; Seneca et al., 2006; Shotbolt et al., 2012; Gallezot et al., 2014). The 92% to 278% larger regional fenfluramine-induced effect on [11C]Cimbi-36 binding than on [11C]MDL 100907 binding in the neocortex is comparable with the 42% to 153% larger regional amphetamine-induced decreases in radioligand binding using different dopamine D/D
3 receptor agonist radioligands in comparison with [11C]raclopride. The results are thus consistent with the view that agonist radioligands are more sensitive to changes in neurotransmitter concentrations than antagonists.

It is worth noting that, in contrast to [11C]MDL 100907 (Pehek et al., 2006), [11C]Cimbi-36 has a relatively poor selectivity for the 5-HT
2A receptor subtype. The affinity (K) is 0.5 to 0.8 nM for the 5-HT
2A receptor, 0.5 nM for the 5-HT
2C receptor, and 1.7 nM for the 5-HT
2A receptor (Ettrup et al., 2011). We previously reported that in the monkey brain, the relative binding of [11C]Cimbi-36 to [11C]MDL 100907 in several regions, including amygdala, hippocampus, thalamus, and midbrain, is higher than in neocortical regions. These differences are most likely attributable to the binding of [11C]Cimbi-36 to 5-HT
2A receptors located either in these regions or in the adjacent choroid plexus (Finnema et al., 2014). Interestingly, similar patterns were also observed in the human brain (Ettrup et al., 2016). Nonetheless, the relative effect of fenfluramine, measured as the ratio of [11C]Cimbi-36 ΔBP
ND to...
### Table 1. Effect of Fenfluramine (FEN) on Regional Binding Potential ($BP_{ND}$) Values (n = 3)

| Region | $[^{11}C]MDL$ 100907 | | $[^{11}C]Cimbi$-36 | | $[^{11}C]AZ10419369$ |
|--------|-----------------|-----------------|-----------------|-----------------|-----------------|
|        | Baseline | FEN | $\Delta BP_{ND}$ (%) | Baseline | FEN | $\Delta BP_{ND}$ (%) | Baseline | FEN | $\Delta BP_{ND}$ (%) |
| Put    | 1.05 | 0.94 | -10.3 | 0.69 | 0.61 | -11.4 | 0.78 | 0.44 | -46.5* |
| CN     | 1.15 | 0.93 | -17.4 | 0.86 | 0.53 | -38.0* | 0.65 | 0.37^ | -46.1* |
| VS     | 1.16 | 0.91 | -21.4 | 0.80^ | 0.56^ | -30.4* | 1.36 | 0.66^ | -51.9** |
| FC     | 3.53 | 2.39 | -29.9 | 1.73 | 0.83 | -51.3* | 0.78 | 0.51 | -35.2 |
| PC     | 2.95 | 1.98 | -30.0 | 1.44 | 0.68 | -51.8* | 0.67 | 0.40 | -42.6* |
| TC     | 2.95 | 2.05 | -28.9* | 1.79 | 0.80 | -54.6* | 0.72 | 0.42 | -42.3* |
| OC     | 2.47 | 1.73 | -26.1 | 1.17 | 0.45 | -62.2** | 1.17 | 0.51 | -58.2* |
| ACC    | 3.46 | 2.41 | -29.3* | 2.14 | 1.03 | -51.1* | 0.91 | 0.59 | -37.0* |
| Amyg   | 1.02 | 0.76 | -25.2 | 0.86 | 0.54^ | -36.6* | 1.05 | 0.55 | -47.6* |
| HC     | 1.27 | 0.85 | -29.8 | 1.03 | 0.49 | -51.5** | 0.63 | 0.37^ | -41.3* |
| Thal   | 0.78 | 0.72 | -8.2* | 0.70 | 0.52 | -25.8** | 0.94 | 0.49 | -48.1* |
| MB     | 0.84^ | 0.67 | -16.3 | 0.66^ | 0.37^ | -43.3** | 0.92 | 0.50^ | -46.3* |
| WB     | 2.03 | 1.46 | -25.8 | 1.19 | 0.60 | -49.4** | 0.69 | 0.38 | -46.3* |

**Abbreviations:** ACC, anterior cingulated cortex; Amyg, amygdala; $\Delta BP_{ND}$ (%) = \frac{[BP_{ND, Pretreatment} - BP_{ND, Baseline}] \times 100}{BP_{ND, Baseline}}; CN, caudate nucleus; FC, frontal cortex; HC, hippocampus; MB, midbrain; OC, occipital cortex; PC, parietal cortex; Put, putamen; TC, temporal cortex; Thal, Thalamus; VS, Ventricle striatum; WB, whole brain.

**Data presented as mean (n = 3).**

Identifiability (%SE) = \frac{\text{Standard error of } BP_{ND, \text{estimation}}}{BP_{ND, \text{estimation}}} \times 100.

*15% ≥ mean identifiability (%SE) > 10%, **20% ≥ mean %SE > 15%.

*P < .05, **P < .01 (1-tailed) by paired t test.

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**Figure 2.** Magnetic resonance images (MRI) and corresponding coregistrated positron emission tomography (PET) summation images (average of frames from 9 to 123 min) of $[^{11}C]MDL$ 100907, $[^{11}C]Cimbi$-36, and $[^{11}C]AZ10419369$ during baseline and post-fenfluramine (FEN) conditions in NHP3. (a) Axial view of images at the level of caudate nucleus. (b) Axial view of images at the level of amygdala/hippocampus; note the difference in the range of the standardized uptake values (SUVs) color bars for the different radioligands. SUVs were calculated from the radioactivity concentration as [kBq/cm$^3$] / (radioactivity injected [MBq] / body weight [kg]). ACC, anterior cingulate cortex; Amyg, amygdala; CN, caudate nucleus; FC, frontal cortex; HC, hippocampus; PC, parietal cortex; OC, occipital cortex; TC, temporal cortex.
[^11C]MDL 100907 ∆BP\textsubscript{ND} was in these 4 regions similar to the ratio in neocortical regions where[^11C]Cimbi-36 binds selectively to the 5-HT\textsubscript{2A} receptors (Finnema et al., 2014). These observations for the neocortical regions suggest that there is a limited contribution of 5-HT\textsubscript{2C} receptor binding to the higher 5-HT sensitivity of[^11C]Cimbi-36 when compared with[^11C]MDL 100907.

The concept of G-protein coupled receptors being in high- and low-affinity states was originally based on studies in vitro (De Lean et al., 1980; Finnema et al., 2010a). In the present study, the fraction of 5-HT\textsubscript{2A} receptors in the high-affinity state in vivo was estimated by dividing the[^11C]MDL 100907 ∆BP\textsubscript{ND} by the[^11C]Cimbi-36 ∆BP\textsubscript{ND} (supplementary Methods). This approach was previously applied to dopamine D\textsubscript{2}/D\textsubscript{3} receptor agonist radioligands and[^11C]raclopride (Narendran et al., 2004, 2010; Seneca et al., 2006; Shotbolt et al., 2012). For reliable estimation of the fraction in vivo, we recommend using neocortical regions, where[^11C]Cimbi-36 binding is specific to 5-HT\textsubscript{2A} receptors (Finnema et al., 2014) and where the test-retest variability is favorable for both radioligands (Talbot et al., 2012; Ettrup et al., 2016). The present in vivo estimates should be taken with caution, since they were based on data obtained in only 3 NHPs but may support the view that high- and low-affinity states are valid concepts also in vivo.

[^11C]AZ10419369 is one of the most sensitive radioligands for endogenous 5-HT (Paterson et al., 2010; Finnema et al., 2015). In addition to high reduction (∼50%) in[^11C]AZ10419369 binding to...
the 5-HT<sub>1A</sub> receptor after administration of fenfluramine 5 mg/kg, the binding was also demonstrated to be sensitive to smaller changes in 5-HT concentration such as after administration of selective serotonin reuptake inhibitors (Finnema et al., 2010b, 2012; Nord et al., 2013). The present study demonstrates that the 5-HT sensitivity of [11C]Cimbi-36 binding is similar to that of [11C]AZ10419369. However, due to the relatively high expression of 5-HT<sub>1A</sub> receptors in neocortical and limbic regions (Paterson et al., 2010), [11C]Cimbi-36 may be advantageous to [11C]AZ10419369 for examination of pathophysiology and treatment effects on serotonergic neurotransmission in those regions.

A limitation of the present study is that fenfluramine and norfenfluramine have been reported to bind to 5-HT<sub>1A</sub> receptors and the potential contribution of direct occupancy, resulting in a decrease in radioligand binding, should therefore be carefully considered (Tyacke and Nutt, 2015). In the present study, the plasma concentrations of fenfluramine and norfenfluramine were measured to estimate the direct occupancy effects (supplementary Methods). The estimated occupancy levels (supplementary Table 2) for the 5-HT<sub>1A</sub> receptor, the 5-HT<sub>2B</sub> receptor, and the 5-HT<sub>2C</sub> receptor (5-13%) were much lower than the observed reductions in radioligand binding (35%-62%). Although the 5-HT<sub>2B</sub> receptor occupancy was estimated to be relatively high (50%), the low and restricted expression of 5-HT<sub>2C</sub> receptors in brain (Nichols and Nichols, 2008) suggests that this binding would have negligible effect on [11C]Cimbi-36 binding in the regions examined. In conclusion, a major proportion of the ΔB<sub>P</sub><sub>P</sub><sub>2</sub> induced by fenfluramine administration can be attributed to 5-HT<sub>1A</sub> release and is not likely to represent drug occupancy at the target receptor.

Another potential limitation of the current PET study is the use of anesthesia. Ketamine and isoflurane have previously been shown not to affect 5-HT<sub>1A</sub> receptor binding of [3H]altanserin in the rodent brain (Elfving et al., 2003). Anesthetic doses of ketamine have however been reported to increase [11C]AZ10419369 binding in NHP brain, but had no effect on fenfluramine-induced decreases in [11C]AZ10419369 binding (Yamanaka et al., 2014). Interestingly, ketamine/xylazine and isoflurane have been reported to elevate the dopamine sensitivity of dopamine D<sub>2</sub>/D<sub>3</sub> receptor agonist radioligands in NHPs and rats, respectively, while isoflurane did not affect [3H]raclopride binding (Ohba et al., 2009; McCormick et al., 2011). However, the increased dopamine sensitivity of dopamine D<sub>2</sub>/D<sub>3</sub> receptor agonist vs antagonist radioligands was confirmed in awake human studies (Narendran et al., 2010; Shotbolt et al., 2012). Future studies to compare the 5-HT sensitivity of [11C]Cimbi-36 and [11C]MDL 100907 in awake humans are warranted to exclude potential anesthetics effects.

The present results may also serve as a starting point for translation to humans, as the NHP brain has higher similarity to the human brain than the pig brain (Capitanio and Emborg, 2008). In one previous study, dexfenfluramine (40 or 60 mg p.o.) was shown to reduce [3H]altanserin antagonist binding to the 5-HT<sub>1A</sub> receptor in human volunteers (Quednow et al., 2012). Following safety considerations, the proposed maximal dose of dexfenfluramine suitable for human use was 1 mg/kg p.o. (Quednow et al., 2012), which is lower than the 5 mg/kg used in the present study. Therefore, it might be interesting to evaluate the effect of a lower dose of fenfluramine (1 mg/kg) on [11C]Cimbi-36 binding in NHPs in comparison with current results. Nonetheless, since 5 mg/kg reduced [11C]Cimbi-36 by 55%, it may be anticipated that [11C]Cimbi-36 binding may be sensitive to 5-HT<sub>1A</sub> release induced also by dexfenfluramine 1 mg/kg in future human studies.

In conclusion, our results support that [11C]Cimbi-36 is a promising radioligand to detect increases in 5-HT concentration in the primate brain. The 5-HT sensitivity of [11C]Cimbi-36 is higher than for [11C]MDL 100907 but similar to [11C]AZ10419369. Future studies are warranted to evaluate the sensitivity of [11C]Cimbi-36 binding to smaller increases in 5-HT concentration and to replicate the current observations in the human brain.

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Statement of Interest

Lars Farde is employed part time at the AstraZeneca, PET Science Center at Karolinska Institutet, Personalized Health Care and Biomarkers. The other authors have no conflicts of interest to declare.

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