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In vivo serotonin-sensitive binding of [^{11}C]CUMI-101: a serotonin 1A receptor agonist positron emission tomography radiotracer

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Positron emission tomography studies of 5-hydroxytryptamine (5-HT)$_{1A}$ receptors have hitherto been limited to antagonist radiotracers. Antagonists do not distinguish high/low-affinity conformations of G protein-coupled receptors and are less likely to be sensitive to intrasynaptic serotonin levels. We developed a novel 5-HT$_{1A}$ agonist radiotracer [^{11}C]CUMI-101. This study evaluates the sensitivity of [^{11}C]CUMI-101 binding to increases in intrasynaptic serotonin induced by intravenous citalopram and fenfluramine. Two *Papio anubis* were scanned, using [^{11}C]CUMI-101 intravenous bolus of 4.5 ± 1.5 mCi. Binding potential ($BP_F = B_{avail}/K_D$) was measured before ($n = 10$) and 20 minutes after elevation of intrasynaptic serotonin by intravenous citalopram (2 mg/kg, $n = 3$; 4 mg/kg, $n = 3$) and fenfluramine (2.5 mg/kg, $n = 3$) using a metabolite-corrected arterial input function. Occupancy was also estimated by the Lassen graphical approach. Both citalopram and fenfluramine effects were significant for BP_F ($P = 0.031$, $P = 0.049$, respectively). The Lassen approach estimated 15.0, 30.4, and 23.7% average occupancy after citalopram 2 mg/kg, 4 mg/kg, and fenfluramine 2.5 mg/kg, respectively. [^{11}C]CUMI-101 binding is sensitive to a large increase in intrasynaptic serotonin in response to robust pharmacological challenges. These modest changes in BP_F may make it unlikely that this ligand will detect changes in intrasynaptic 5-HT under physiologic conditions; future work will focus on evaluating its utility in measuring the responsiveness of the 5-HT system to pharmacological challenges.
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Introduction

Serotonin 1A (5-hydroxytryptamine, 5-HT$_{1A}$) receptor binding measured with *in vivo* imaging has been found to be altered in mood disorders (Parsey *et al*, 2006), anxiety disorders (Judd *et al*, 1994), and some (Abi-Dargham *et al*, 1997), but not all (Frankle *et al*, 2006), schizophrenia studies. All published human imaging studies to date have used an antagonist as the imaging ligand, most commonly [^{11}C]WAY100635. Although initial efforts to develop an agonist tracer for the 5HT$_{1A}$ receptor have not succeeded in primates (Kumar and Mann, 2007), we have recently reported success with a novel agonist 5-HT$_{1A}$ radioligand, [^{11}C]CUMI-101 (Milak *et al*, 2008). A G protein-coupled transmembrane receptor, the 5-HT$_{1A}$ receptor exists in an active or high-affinity state, which is coupled to the G protein, and in an inactive or low-affinity state, which is not coupled to the G protein (Cheney *et al*, 1982). As antagonists bind with equal affinity to both states of the receptor (Lahti *et al*, 1992), antagonists alone cannot detect the proportion of high-affinity or active receptors.

Theory predicts that agonist radiotracers are more likely to be sensitive to changes in intrasynaptic concentrations of the neurotransmitter. This claim has been substantiated in studies of dopamine D_2 receptors, which found that binding of an agonist

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ligand proved to be more sensitive than that of an antagonist to pharmacologically evoked changes in dopamine release in awake mice (Cumming *et al*, 2002) and anesthetized monkeys (Narendran *et al*, 2004).

Both citalopram (Hjorth, 1993; Sharp *et al*, 1989) and fenfluramine (Hoebel *et al*, 1989; Laferrere and Wurtman, 1989) are known to increase endogenous 5-HT release extracellularly. To evaluate the sensitivity of [11C]CUMI-101 to displacement by endogenous serotonin from 5-HT_{1A} receptors, we measured the binding potential ($BP_F = B_{avail}/K_D$) of [11C]CUMI-101 in *Papio anubis* before and after intravenous citalopram or fenfluramine. We hypothesized that the transient increase in intrasynaptic serotonin, in response to citalopram and fenfluramine, will increase occupancy measurable by [11C]CUMI-101 positron emission tomography (PET).

Materials and methods

All animal experiments were carried out with the approval of the Institutional Animal Care and Use Committees from Columbia University Medical Center and New York State Psychiatric Institute.

Chemistry and Radiochemistry

[11C]CUMI-101 was synthesized as described by our laboratory (Kumar *et al*, 2007). In brief, the radiotracer was prepared by radiomethylation of the corresponding desmethyl analog using [11C]CH_3OTf. The final product was purified by high-performance liquid chromatography and a C-18 SepPak (Milford, MA, USA). The radioproduct eluted from the C-18 SepPak (in 1 mL ethanol) was diluted with 9 mL of normal saline, filtered through an aseptically prepared 0.22 μm filter, and used for further studies. A small portion of the product was analyzed with high-performance liquid chromatography for chemical and radiochemical purities, specific activity, and other quality control indices. The average radiochemical yield of [11C]CUMI-101 was 25% end of synthesis (EOS) with a specific activity of 2,600 ± 500 Ci/μmol.

Positron Emission Tomography Studies

A series of [11C]CUMI-101 PET scans were performed in two male *P. anubis* using an ECAT EXACT HR+ scanner (Siemens, Knoxville, TN, USA). Animal anesthesia and preparation were as described previously (Milak *et al*, 2005). Overall, 4.48 ± 1.47 mCi (specific activity: 1.8 ± 0.9 Ci/μmol) of [11C]CUMI-101 was injected as an intravenous bolus over 30 seconds and emission data were collected for 120 minutes in a three-dimensional mode. Plasma samples were collected every 10 seconds for the first 2 minutes using an automated system, and thereafter manually for a total of 34 samples. The challenge experiments used the high-affinity, selective serotonin transporter inhibitor citalopram (3 scans at 2 mg/kg, intravenous and 3 scans at 4 mg/kg, intravenous) or fenfluramine (3 scans at 2.5 mg/kg intravenous) 30 minutes before the second injection of [11C]CUMI-101. Citalopram (Ki > 10,000) and fenfluramine (Ki = 673) or their metabolites have no significant binding affinity to the 5-HT_{1A} receptors (PDSP Database—UNC %U; http://kidb.cwru.edu/pdsp.php). For comparison, seven baseline experiments were used from each animal. For the graphical analysis of occupancy by an antagonist, one scan was used from each animal wherein WAY100635 (a potent 5-HT_{1A} antagonist synthesized in house) was administered intravenously (0.5 mg/kg) 30 minutes before scanning. It must be noted that there is a fundamental difference between occupancy by an antagonist injected intravenously such as WAY100635 and increasing occupancy (relative to baseline) by the endogenous serotonin in response to a pharmacological challenge. Nevertheless, the radiotracer [11C]CUMI-101 may be displaced from receptor binding by either intervention.

Metabolite and Free Fraction Analysis

Six plasma samples were obtained at 2, 4, 12, 30, 60, and 90 minutes during each scan to measure the percentage of unmetabolized [11C]CUMI-101. Free fraction was determined using an ultracentrifugation method as described elsewhere (Gandelman *et al*, 1994; Ginovart *et al*, 2001). (High-performance liquid chromatography column: Phenomenex (Torrance, CA, USA) Prodigy ODS(3) 4.6°—250 mm, 5 μm; mobile phase: acetonitrile: 0.25 mol/L sodium phosphate solution (40:60); flow rate: 2 mL/min; retention time: 6 minutes). The metabolites and free fractions were assayed using a Perkin-Elmer 3″ NaI gamma detector (Perkin-Elmer, Waltham, MA, USA). Data were corrected for background radioactivity and decay. The six metabolite points were fitted with the Hill function $(1-At^B/(t^B+C))$ and weighted using the delta method (Wu *et al*, 2007). This metabolite fit was then used to correct the plasma radioactivity (34 samples), and a 3-exponential function was fit to the metabolite-corrected plasma curve.

Image Processing and Analysis

Positron emission tomography data were reconstructed after transmission-based attenuation correction and model-based scatter correction (Watson *et al*, 1996). The reconstruction filter and estimated image filter were Shepp 0.5 (2.5 full-width half maximum; Siemens/CTI), the Z filter was All Pass 0.4 (2.0 full-width half maximum; Siemens/CTI), and the zoom factor was 4.0, leading to a final image resolution of 5.1 mm full-width half maximum at the center of the field of view (Mawlawi *et al*, 2001).

A T1-weighted magnetic resonance image of the head of both animals was acquired on a GE 1.5-T Signa Advantage system (GE Healthcare Biosciences, Pittsburgh, PA, USA). Regions of interest (ROIs) included the anterior cingulate, amygdala, hippocampus, insular cortex, prefrontal cortex, and temporal cortex and were drawn on coregistered magnetic resonance images; the dorsal raphe nucleus was delineated on baseline PET scans. A part of the cerebellar

hemispheres, excluding areas adjacent to the occipital cortex and the vermis, was the reference region.

V_T values were derived using likelihood estimation in graphical analysis (Ogden, 2003; Parsey *et al*, 2003) of the time–activity curve data and metabolite-corrected arterial input functions (Milak *et al*, 2008). Outcome measures were total volume of distribution (V_T) and binding potential (BP_F = difference in V_T values of an ROI and the reference region divided by the free fraction, f_p). Test–retest variability was calculated as the absolute value of the difference between test and retest values, divided by the mean of the two measurements as reported elsewhere (Milak *et al*, 2008).

Statistical Analysis

Statistical analysis was performed on the BP_F data at the ROI level using a linear mixed-effects model with ROI and dose as fixed effects (separately for citalopram and fenfluramine). The random effects are animal, scan date (nested within animal), and scan (nested within scan date). Response variables were taken to be log-transformed BP_F values. This is to stabilize the variance between regions, to correct for some slight skewness in the measurements, and because our primary hypothesis specifies a proportional change in each ROI.

As we have previously shown, there is no ideal reference region for 5-HT_{1A} scans in baboons (Milak *et al*, 2008). Therefore, occupancy was also evaluated using a graphical approach first described by Lassen *et al* (1995) and recently modified and expanded by Cunningham *et al* (2010). Lassen *et al* showed that graphical representation of $x = V_T^{Base}$ and $y = V_T^{Base} - V_T^{Drug}$ produces a linear relationship with the x intercept equal to V_{ND} and slope of the linear fit equal to the occupancy. As this approach yields a linear relationship between 'x' and 'y' regardless of whether any of the ROIs plotted is a 'true' reference region, it follows that this approach does not require a reference region. This approach provides a reference region independent of the verification of occupancy and an estimate of V_{ND} (x intercept), assuming it is uniform across ROIs. Cunningham *et al* (2010) showed that this linear relationship holds true even when two different drug concentrations are used in lieu of a baseline scan ($x = V_T^{Drug1}$ and $y = V_T^{Drug1} - V_T^{Drug2}$). They also suggested that 'when more than one occupancy scan is available within the same individual, it may be better to constrain all x axis intercepts to be equal as part of the fitting process.' Accordingly, we estimated the 'true' V_{ND} using the near complete block produced by nonradioactive WAY100635 0.5 mg/kg intravenous (published elsewhere (Milak *et al*, 2008); see Figure 1) and used this value to constrain the x axis intercepts of the linear fit in the graphical approach for estimating occupancy.

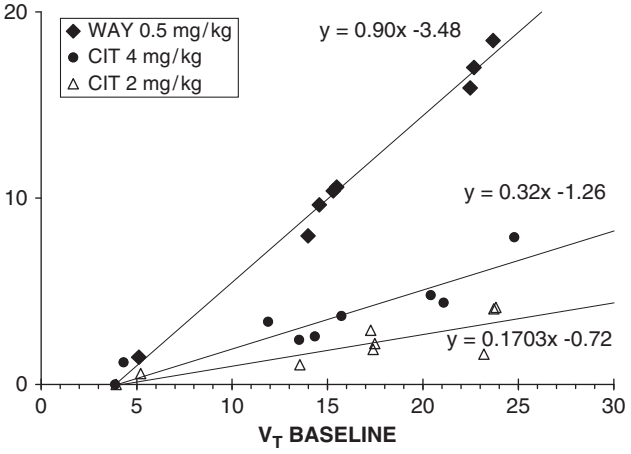

Figure 1 Graphical analysis applied to PET occupancy studies. Data are collected from a single [11C]CUMI-101 study in baboons at baseline and after intravenous administration of a 5-HT_{1A} antagonist WAY100635 at 0.5 mg/kg (diamonds); black circles and triangles represent data at baseline and after intravenous administration of citalopram at 4 mg/kg and 2 mg/kg, respectively (single studies). In all cases, the estimated V_{ND} values (or x axis intercept of the linear fit), were not included in the regression analysis. PET, positron emission tomography; 5-HT_{1A}, 5-hydroxytryptamine$_{1A}$.

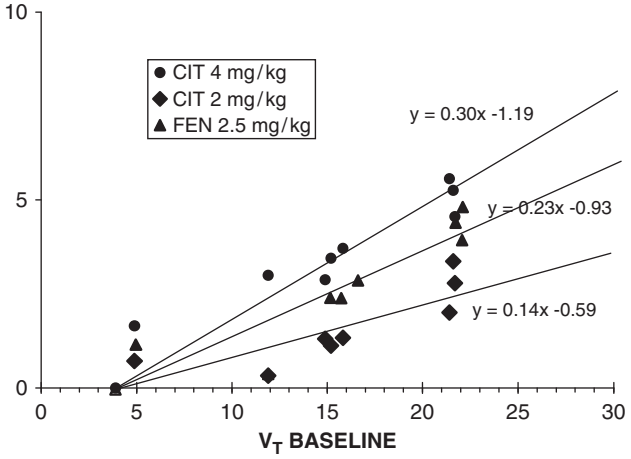

Figure 2 Graphical analysis applied to PET occupancy studies. Data are collected from [11C]CUMI-101 study in baboons at baseline and after intravenous administration of citalopram 4 mg/kg (circles); citalopram 2 mg/kg (diamonds), and fenfluramine 2 mg/kg (triangles), respectively (each data points representing means from three experiments). In all cases, the estimated V_{ND} values were constrained to estimates from the blocks achieved using cold 0.5 mg/kg WAY100635 (see Figure 1). PET, positron emission tomography.

Results

Citalopram

The citalopram dose response (main effect) was significant for BP_F (df = 1, 5; F = 8. 97; $P = 0.031$). The interaction terms between citalopram dose and ROI remained nonsignificant (df = 6, 84; F = 0.42; $P = 0.87$).

Figure 1 shows the application of the Lassen graphical approach to representative single experiments, whereas Figure 2 shows the application of this approach to the average of all the experiments at

each dose level. The Lassen graphical approach based on V_T yielded an average occupancy of 14.98% (see Figure 2) in response to 2 mg/kg citalopram and an average occupancy of 30.39% (see Figure 2) in response to 4 mg/kg citalopram (relative to the unknown basal occupancy).

Fenfluramine

The effect of fenfluramine administration was significant (df = 1, 2; F = 18.8; P = 0.049). There was no significant interaction between fenfluramine dose and ROI (df = 6, 24; F = 1.37; P = 0.27). The Lassen graphical approach based on V_T yielded an average occupancy of 23.74% in response to 2.5 mg/kg fenfluramine.

Clearance of [11C]CUMI-101 from the plasma after citalopram or fenfluramine pretreatment, estimated as the area under the fitted metabolite-corrected plasma input function divided by the injected dose, was unchanged (df = 1, 5; F = 3.68; P = 0.11; df = 1, 2; F = 0.006; P = 0.95, respectively).

The plasma free fraction (f_P) was also unaffected by citalopram or fenfluramine pretreatment (df = 1, 5; F = 0.03; P = 0.86; df = 1, 2; F = 1.34; P = 0.37, respectively).

Although cerebellar V_T values were small (see Figure 3, top panel), reduction was also seen in the cerebellum in response to citalopram: V_T was reduced 15% ± 12% by 2 mg/kg and 34% ± 8% by 4 mg/kg citalopram pretreatment. Therefore, any measurements of change in BP_F, which is dependent on a 'true' reference tissue, are underestimates. Nevertheless, the dose response of BP_F to citalopram is significant and well outside the range of test–retest variability of 11% ± 5% (for measures of BP_F) (Milak *et al*, 2008).

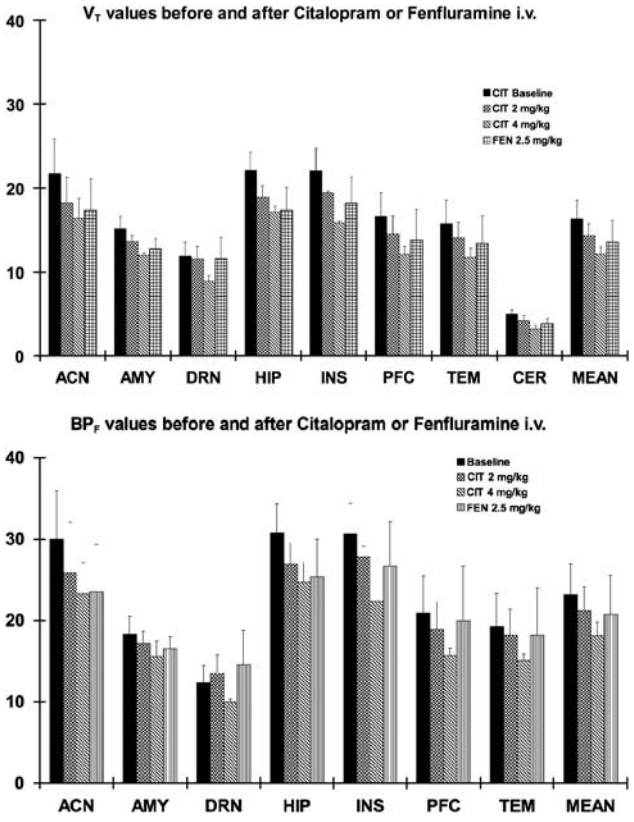

Figure 3 Mean V_T (top) and BP_F (bottom) of [11C]CUMI-101 after citalopram (2 or 4 mg/kg) and fenfluramine (2.5 mg/kg) intravenously. These values were derived from the LEGA model with scan duration of 100 minutes. Error bars represent the s.d. LEGA, likelihood estimation in graphical analysis.

Discussion

Pretreatment with either citalopram or fenfluramine intravenous resulted in a decrease in [11C]CUMI-101 binding (BP_F) in 5-HT_{1A}-rich regions of the brain, without any observable changes in the metabolite-corrected arterial input function or free fraction.

[11C]CUMI-101 Displacement by Endogenous 5-Hydroxytryptamine

BP_F as the Outcome Measure: Changes in BP_F values after citalopram pretreatment show a significant dose–response effect. Changes are modest in response to 2 mg/kg intravenous citalopram pretreatment when averaged across all ROIs. However, the 4 mg/kg dose produces a greater reduction in both V_T and BP_F across all ROIs, including the cerebellum. This is consistent with our earlier finding that the cerebellum is not completely devoid of 5-HT_{1A} receptors (Parsey *et al*, 2005). Although the cerebellar V_T is small, this decrease in V_T of the reference region introduces a bias in the estimation of BP_F that causes an underestimation of the change in BP_F in ROIs. The average cerebellar V_T before WAY100635 block across animals was 5.35 ± 0.3; the average cerebellar V_T across animals after WAY100635 block was 3.89 ± 0.36; the average V_{ND} estimated by the Lassen graphical approach across animals was 3.91 ± 0.03. This suggests that there is a ~27.3% difference between cerebellar V_T and V_{ND}, as estimated by the Lassen plot, in baboons.

V_T/f_p as the Outcome Measure: To provide an outcome measure free of this bias, we repeated the analysis using V_T/f_p, which is the closest approximation of B_{avail}/K_D that can be calculated in the absence of a 'true' reference region (Theodore *et al*, 2007). Using V_T/f_p, both citalopram and fenfluramine dose effects remained significant (df = 1, 5, F = 36.10, P = 0.002; df = 1, 2, F = 25.83, P = 0.037, respectively). Therefore, we conclude that administration of both citalopram and fenfluramine reduces 5-HT_{1A} binding in *P. anubis*, despite both a possible anesthesia effect (Whittington and Virag, 2006) and the reduction of V_T in the reference region, which acts to diminish the

percentage change measured in BP_F (see limitations below). The corresponding, but greater reduction in V_T/f_p provides convincing support to this conclusion.

Lassen Graphical Approach

In addition, these results are supported by occupancy estimates obtained by the Lassen graphical approach in which we found a doubling of the receptor occupancy by serotonin after 4 mg/kg compared with 2 mg/kg of citalopram. This approach is less likely to be affected by bias and measurement noise, as it requires far fewer assumptions than using either one of the previous outcome measures (i.e., the method does not require a measurement of V_{ND} or f_p). The Lassen graphical approach provides a reliable estimate of occupancy and V_{ND} (x intercept), even if there is no 'true' reference region devoid of the receptor being investigated.

Previous Attempts to Measure Endogenous 5-Hydroxytryptamine Release

Similar studies using antagonist ligands [11C]WAY100635 or [18F]MPPF report no reliably measurable effects on binding in response to pharmacological challenges known to stimulate the release of endogenous serotonin. In baboons, using 1.5 mg/kg of amphetamine intravenously or fenfluramine per os, we have previously reported no decrease in [11C]WAY100635 binding (Parsey *et al*, 1998). These data as well as data reported in the current study may be confounded by an anesthetic affect which has been described previously (Whittington and Virag, 2006). Isoflurane is reported to reduce serotonin release and may mitigate the effects of citalopram or fenfluramine.

It is likely that only a robust increase in intrasynaptic serotonin can be detected by such methods. *In vivo* studies in rats using microdialysis (Hume *et al*, 2001*b*) report that doses of fenfluramine (10 mg/kg intravenous), which resulted in an $\sim$15-fold increase in extracellular serotonin in the hippocampus, produce a modest ($<$20%) decrease in hippocampal [11C]WAY100635 binding. In these same experiments, no changes in cortex and raphe binding were found, despite an approximately five-fold increase in extracellular serotonin measured by microdialysis.

Serotonin releasers and depleters, such as reserpine, do not affect [11C]WAY100635 binding in the hippocampus or cortex of male Sprague–Dawley rats (Maeda *et al*, 2001). Similarly, in the human brain, [11C]WAY100635 binding was unaffected by tryptophan depletion and augmentation (Rabiner *et al*, 2002).

Few studies have been carried out with other radioligands. One study (Mathis *et al*, 1995) has shown that fenfluramine decreases the specific binding of [11C]WAY100635 in rats and rhesus monkeys. However, it has been argued (Zimmer *et al*, 2002) that these results were of little practical value as the dose of fenfluramine was high (8 mg/kg, intravenous) and the resulting displacement low ($<$20%).

In the past, we found that administration of fenfluramine failed to decrease [11C]WAY100635 binding (Parsey *et al*, 1999). In contrast, others report 10% to 20% reduction in the specific binding of [11C]WAY100635 in the hippocampus of rats, but only after large doses of fenfluramine treatment (10 mg/kg intraperitoneally) (Hume *et al*, 2001*a*). No changes in BP_F in either the prefrontal cortex or midbrain raphe were observed. These authors argue that these minimal effects are consistent with a low baseline occupancy of the 5-HT_{1A} receptor by 5-HT *in vivo*, suggesting that only a large change in endogenous agonist concentration would affect radioligand binding. However, the validity of this claim has since been refuted (Zimmer *et al*, 2002). In contrast, our results show that [11C]CUMI-101 binding (BP_F) is reduced by an average of 23.74% in response to a much lower dose of fenfluramine (2.5 mg/kg). The studies showing direct competition between endogenous 5-HT and [18F]-MPPF were carried out mostly in rats and radioactivity was measured by a β-sensitive probe, not PET. Unfortunately, when these paradigms were tested in nonhuman primates (Udo de Haes *et al*, 2006) and humans using PET, the antagonist [18F]-MPPF did not show measurable binding changes (Udo de Haes *et al*, 2002; Udo de Haes *et al*, 2005; Udo de Haes *et al*, 2006).

[11C]CUMI-101 appears more vulnerable to displacement by endogenous serotonin than several antagonists ligands tested to date (but perhaps not as much as would have been expected on the basis of the agonist competition model alone).

Limitations

This study has been carried out in animals under general anesthesia using isoflurane which, as mentioned above, is reported to reduce serotonin release. This study is also limited by small sample size (only two animals were used). However, this design allowed us to show that the findings were consistently reproduced in each animal more than once and that the occupancy results show a convincing dose–response effect.

Future Directions

Future work will adapt these studies to humans to evaluate levels of serotonin release in health and disorders involving the serotonin system. In addition to testing the sensitivity of this ligand to increases in serotonin, the approach can evaluate effects of acute tryptophan depletion that reduces serotonin levels (and is considerably more tolerable to humans than

reserpine). Pharmacokinetic studies of citalopram in humans (Fredricson Overo, 1982) showed that the steady-state plasma levels in patients range from 95 to 720 nmol/L (~40 to 300 ng/mL) at doses of 30 to 60 mg per day. The mean level was 245 nmol/L (~100 ng/mL) at the standard dose of 40 mg daily (Fredricson Overo, 1982). In comparison, in the current study we found that citalopram plasma levels after pretreatment with 4 mg/kg were ~238 ng/mL (~590 nmol/L) when averaged throughout the PET scans. This suggests that comparable citalopram plasma levels are readily achievable and therefore these experiments may be reproducible in human subjects.

Summary

This study is the first report of a 5-HT_{1A} agonist PET radioligand in primates that can measure robust increases in intrasynaptic endogenous serotonin. If replicated in humans, this radioligand may open the door to studying dynamic changes in intrasynaptic 5-HT levels in a wide range of experimental conditions and psychiatric disorders, as well as studies related to drug development and pharmacotherapy.

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Conflict of interest

The authors declare no conflict of interest.

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