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Evaluation in Monkey of Two Candidate PET Radioligands, [^{11}C]RX-1 and [^{18}F]RX-2, for Imaging Brain 5-HT $_4$ Receptors

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Abstract

The serotonin subtype-4 (5-HT $_4$) receptor, which is known to be involved physiologically in learning and memory, and pathologically in Alzheimer's disease, anxiety and other neuropsychiatric disorders – has few radioligands readily available for imaging in vivo. We have previously reported two novel 5-HT $_4$ receptor radioligands, namely [*methoxy*- ^{11}C](1-butylpiperidin-4-yl)methyl 4-amino-3-methoxybenzoate; [^{11}C]RX-1) and the [^{18}F]3-fluoromethoxy analog ([^{18}F]RX-2), and in this study we evaluated them by PET in rhesus monkey. Brain scans were performed at baseline, receptor preblock or displacement conditions using SB 207710, a 5-HT $_4$ receptor antagonist, on the same day for [^{11}C]RX-1 and on different days for [^{18}F]RX-2. Specific-to-nondisplaceable ratio (BP_{ND}) was measured with the simplified reference tissue model from all baseline scans. To determine specific binding, total distribution volume (V_{T}) was also measured in some monkeys by radiometabolite-corrected arterial input function after ex vivo inhibition of esterases from baseline and blocked scans. Both radioligands showed moderate to high peak brain uptake of radioactivity (2–6 SUV). Regional BP_{ND} values were in the rank order of known 5-HT $_4$ receptor distribution with a trend for higher BP_{ND} values from [^{18}F]RX-2. One-tissue compartmental model provided good fits with well identified V_{T} values for both radioligands. In the highest 5-HT $_4$ receptor density region, striatum, 50–60% of total binding was specific. The V_{T} in receptor-poor cerebellum reached stable values by about 60 min for both radioligands indicating little influence of radiometabolites on brain signal. In conclusion, both [^{11}C]RX-1 and [^{18}F]RX-2 showed positive attributes for PET imaging of brain 5-HT $_4$ receptors, validating the radioligand design strategy.

Keywords

PET; Imaging; 5-HT $_4$; Radioligand; Carbon-11; Fluorine-18; Brain

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INTRODUCTION

Serotonin (5-hydroxytryptamine; 5-HT), is a major neurotransmitter acting upon several receptor subtypes in brain, among which is the well characterized serotonin subtype 4 (5-HT₄) receptor (Eglen et al., 1995, 1996; Bockaert et al., 2008). This G-protein-coupled receptor is positively linked to adenylyl cyclase (Dumuis et al., 1988) with influence over acetylcholine (Mohler et al., 2007), dopamine (Alex and Pehek, 2007) and serotonin release (Ge et al., 1996). The distribution of 5-HT₄ receptors in human brain is postsynaptic and is relatively dense in basal ganglia and hippocampal formation, less dense in neocortex and absent in cerebellum (Reynolds et al., 1995; Varnäs et al., 2003). Brain distribution and other evidence suggest that 5-HT₄ receptors have functions in normal cognition, learning and memory (Meneses et al., 1997; Matsumoto et al., 2001; Moser et al., 2002). Perturbations to 5-HT₄ receptors have been implicated in some neuropsychiatric disorders, including Alzheimer's disease (Lezoualc'h et al., 2007), depression (Warner-Schmidt et al., 2009), and eating disorders (Rybkin II et al., 1997; Jean et al., 2007). 5-HT₄ receptors are now considered a possible target for new drugs to treat such disorders (Lucas 2009; Bockaert et al., 2011). Thus, for example, administration of 5-HT₄ agonists in animal models of Alzheimer's disease increases acetylcholine release and improves memory and cognition (Cho et al., 2007). Additionally, activation of 5-HT₄ receptors has been found to produce a rapid antidepressant response in rodent models of depression (Lucas et al., 2007).

Positron emission tomography (PET) has emerged as a powerful tool for neuropsychiatric research and drug development when harnessed with biochemically-specific radiotracers. In particular, radioligands for neurotransmitter receptors have shown value not only for enhancing our understanding of neuropsychiatric disorders (Wong et al., 2007) but also for evaluating target engagement by experimental therapeutics (Gibson et al., 2008). Successfully developed PET radioligands for imaging serotonin subtype 1A and subtype 2A receptors have served such purposes, but until recently radioligands for imaging brain 5-HT₄ receptors have been lacking (Paterson et al., 2013). The potential to image 5-HT₄ receptors in living brain was first shown in monkey with single photon emission computed tomography and the radioligand [¹²³I]SB 207710 (Pike et al., 2003) (Figure 1). More recently, promising PET radioligands have been developed based on the same core structure as SB 207710, namely [¹¹C]SB 207145 (Kornum et al., 2009), [¹⁸F]MNI-698 (Caillé et al., 2013), and [¹⁸F]MNI-699 (Caillé et al., 2013) (Figure 1). Only [¹¹C]SB 207145 has so far advanced to use in human subjects (Gee et al., 2008; Marner et al., 2009; Madsen et al., 2011a,b), including studies of Alzheimer's disease (Madsen et al. 2011c). Each of these radioligands may be susceptible to rapid ester hydrolysis to give a radioactive alcohol that might enter brain and possibly confound accurate quantification of 5-HT₄ receptors in vivo. Therefore, we had earlier explored developing 5-HT₄ radioligands with a radiolabel on the other side of the ester group with the expectation that ester hydrolysis would initially give a labeled carboxylic acid as a primary radiometabolite and that this radiometabolite would be well excluded from brain because of predominant ionization at physiological pH (Xu et al., 2010). Our medicinal chemistry showed that [¹¹C]RX-1 (Figure 1) possessed attractive *prima facie* features (Pike 1993; Laruelle et al., 2003; Patel and Gibson, 2008) as a candidate PET radioligand, including antagonist action, high 5-HT₄ receptor affinity, high binding

selectivity, moderate lipophilicity and amenability to labeling. The fluoromethyl analog, [^{18}F]RX-2 (Figure 1), also possessed many of these features, except an apparently lower 5-HT₄ receptor affinity (Xu et al, 2010). In this study, we aimed to evaluate both [^{11}C]RX-1 and [^{18}F]RX-2 as PET radioligands in rhesus monkey. Each radioligand was found to give moderate but stable 5-HT₄ receptor-specific signal, thereby validating the radioligand design strategy.

MATERIALS AND METHODS

Materials

Sodium azide, sodium fluoride and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma-Aldrich (Milwaukee, WI). (1-Butylpiperidin-4-yl)methyl 8-amino-7-chloro-2,3-dihydrobenzo[*b*][1,4]dioxane-5-carboxylate (SB 207710), phenol precursor for radiolabeling (1-butylpiperidin-4-yl)methyl 4-amino-3-hydroxybenzoate), RX-1 (1-butylpiperidin-4-yl)methyl 4-amino-3-(methoxy)benzoate), and RX-2 (1-butylpiperidin-4-yl)methyl 4-amino-3-(fluoromethoxy)benzoate) were prepared as described previously (Xu et al., 2010).

General Methods

γ -Radioactivity from ^{11}C and ^{18}F was measured using a calibrated dose calibrator (Atomlab 300; Biodex Medical Systems; Shirley, NY). Radioactivity measurements were corrected for physical decay. All radiochemistry was performed in lead-shielded hot-cells with automated apparatus for personnel protection from radiation.

Pharmacological Screening

RX-1, RX-2 and SB 207710 were submitted to the National Institute of Mental Health Psychoactive Drug Screening Program (NIMH-PDSP) for assay of binding affinity to human recombinant 5-HT₄ receptors using [^3H]GR 113808 as reference radioligand. RX-2 was also submitted for assay of binding affinity at a wide range of other human recombinant receptors and binding sites, and also the rat brain benzodiazepine site. Detailed assay protocols are available at the NIMH-PDSP web site (<http://pdsp.cwru.edu>). RX-1 had been similarly screened previously (Xu et al., 2010).

Radioligand Synthesis

[^{11}C]RX-1 was prepared essentially as described previously (Xu et al., 2010). Thus, [^{11}C]iodomethane (14.3–19.0 GBq) was trapped in a V-vial (0.9 mL) containing the phenol precursor (0.53–0.63 mg), TBAH solution (0.5M, 5 μL ; 1.5 eq.), and DMF (300 μL). Alternatively, [^{11}C]methyl triflate (5.4–10.2 GBq) was trapped in a V-vial (0.9 mL) containing the phenol precursor (0.15–0.8 mg), NaOH solution (0.5M, 3 μL ; 3.2–0.6 eq.), and acetonitrile (300 μL). In each method, the sealed reaction mixture was heated at 80 °C for 5 min and then diluted with HPLC mobile phase [MeCN–100 mM HCOONH₄ (2: 3 v/v); 500 μL]. [^{11}C]RX-1 (t_R = 8.7 min) was isolated with radio-HPLC on a Gemini-NX C-18 column (5 μm , 4.6 $\times$ 250 mm) eluted with mobile phase at 3 mL/min. [^{11}C]RX-1 was collected, formulated for intravenous injection in saline (10 mL), and analyzed by reverse phase radio-HPLC, as described previously (Xu et al. 2010).

[¹⁸F]RX-2 was prepared by treatment of the corresponding phenol precursor with [¹⁸F]fluoromethyl bromide-*d*₂ (Chin et al. 2006), essentially as described previously (Xu et al. 2010). Thus, [¹⁸F]fluoromethyl-*d*₂ bromide (2.2–3.7 GBq) was trapped in a V-vial (1 mL) containing the phenol precursor (0.23–0.33 mg), NaOH solution (0.5M, 4.6–6.6 μL, 3 eq.) and acetonitrile (400 μL). The sealed reaction mixture was heated at 100 °C for 30 min and then diluted with water (0.8 mL). [¹⁸F]RX-2 (*t*_R = 26 min) was isolated with radio-HPLC on a Gemini-NX C-18 column (5 μm, 10 × 250 mm) eluted with mobile phase (MeCN-100 mM HCOONH₄ (65: 35 v/v) at 3 mL/min. Alternatively, a Prodigy C-18 column (10 μm, 10 × 250 mm) eluted with 70: 30 v/v at 7 mL/min resulted in a similar HPLC separation and retention time. [¹⁸F]RX-2 was collected, formulated for intravenous injection in 10% ethanol/saline (v/v), and analyzed by reverse phase radio-HPLC, as described previously (Xu et al. 2010).

Measurement of *LogD*

The values for the distribution coefficients of [¹¹C]RX-1 and [¹⁸F]RX-2 between *n*-octanol and sodium phosphate buffer (0.15M, pH 7.4) were determined at room temperature by a method described previously (Zoghbi et al., 2012).

Measurement of Plasma Free Fractions

Plasma free fractions (*f*_p) were determined in triplicate for [¹¹C]RX-1 and [¹⁸F]RX-2 in arterial plasma sampled from two monkeys, by a method described previously (Gandelman et al., 1994).

Stabilities of Radioligands in Buffer In Vitro

Formulated radioligand solution (5 μL; 18 μCi) was added to sodium phosphate buffer (0.15M; pH 7.4; 0.5 mL) and incubated at room temperature for at least 2.5 h. A sample was then analyzed with HPLC on a Nova-Pak C18 column (4 μm, 100 mm × 8 mm; Waters Corp., Columbia, MD) housed in a radial compression module (RCM 100) and eluted with MeOH: H₂O: Et₃N (75: 25 : 0.1 v/v) at 2.0 mL/min. Subsequent injection of methanol (2 mL) onto the HPLC column was used to confirm that all injected radioactivity had been recovered during the analysis.

Inhibition of Radioligand Hydrolysis in Whole Blood and Plasma In Vitro

The following inhibitor solutions were prepared: 1) aqueous sodium azide (41.7 g/100 mL); 2) aqueous sodium fluoride (4 g/100 mL); and 3) PMSF in DMSO (87.1 mg/mL) mixed with an equal volume of the prepared sodium fluoride solution. Anticoagulated (EDTA) whole blood was drawn from untreated rhesus monkeys. A sample of each inhibitor solution (5 μL for NaF; 5 μL for PMSF solution) was mixed with whole blood or plasma (0.5 mL). Formulated [¹¹C]RX-1 solution (5 μL; 18 μCi) was then added to each sample. All mixtures were incubated at room temperature for 30 min. Then a sample (50 μL) was removed from each mixture and placed in an Eppendorf tube containing acetonitrile (300 μL), mixed and centrifuged. Precipitates were counted in an automatic γ-counter to calculate the radioactivity recovered into acetonitrile. The supernatant liquid was then analyzed by radio-HPLC as described above.

Analysis of Radiometabolites in Plasma

During PET scanning sessions at baseline in two monkeys with [^{11}C]RX-1 and in two monkeys with [^{18}F]RX-2, arterial blood samples, anticoagulated with heparin, were drawn periodically and measured for radioactivity in a γ -counter. The samples were centrifuged, and the plasma was separated. A plasma sample of known volume (50–200 μL) was measured for radioactivity. Another sample of plasma of known volume (300–450 μL) was mixed with acetonitrile (0.7 mL) and then centrifuged (10,000g). The precipitates were counted for radioactivity to allow calculation of extraction efficiency. The supernatant liquid was analyzed by radio-HPLC on a Novapak C18 column (4 μm , 100 $\times$ 8 mm; Waters Corp.) housed in a radial compression module (RCM-100) and eluted at 2.0 mL/min with MeOH: H₂O: Et₃N (65: 35: 0.1 by vol.), as described previously (Zoghbi et al., 2006). Full recovery of radioactivity from each HPLC analysis was confirmed by subsequent injection of methanol (2 mL) and observation of no more radioactivity elution from the column. The composition of decay-corrected radioactivity in plasma, in terms of the percentages of radioligand and its radiometabolites, was calculated from the acquired data. Radioactivity levels in blood and plasma were expressed as a standardized uptake value ($\text{SUV} = [(\% \text{ injected activity}/\text{cm}^3) \times \text{body weight (g)}]/100$).

Monkey PET Experiments

Experiments were performed in a total of 5 male rhesus monkeys (*Macaca mulatta*) in compliance with *Guide for the Care and Use of Laboratory Animals* (National Academy Press, 1996) and were approved by the NIMH Animal Care and Use Committee. Anesthesia was induced with ketamine (10 mg/kg, i.m.) and then maintained with 1.0–3.0% isoflurane and 98.4% O₂. Head was firmly fixed by gauze and tapes to the camera bed holder and positioned by laser guidance. Throughout the experiment electrocardiogram, heart and respiration rates were monitored, and body temperature was kept between 37.0 and 37.5°C. Radioligands were administered as bolus intravenous injections. PET images were acquired with a microPET Focus 220 scanner (Siemens Medical Solution; Knoxville, TN) for 120 min in 33 frames, with frame durations ranging from 30 s to 5 min. All PET images were corrected for attenuation and scatter.

Experiments with [^{11}C]RX-1—Three monkeys (6.0, 6.7, and 12.7 kg) were used in a total of eight scanning sessions. These were comprised of four baseline experiments in which [^{11}C]RX-1 was administered alone, followed in each case at 3 h later after the first radioligand injection with a receptor preblock experiment in the same monkey in which the selective 5-HT₄ antagonist SB 207710 (0.5 mg or 1.0 mg/kg; i.v) was administered at 10 min before a second [^{11}C]RX-1 injection. In two monkeys, arterial samples were collected for metabolite-corrected input function in both baseline and preblocked scans. Injected activities were 171–222 MBq. In the baseline experiments, the specific activities of [^{11}C]RX-1 at time of injection were 61–109 GBq/ μmol .

Experiments with [^{18}F]RX-2—Five monkeys (7.4, 10.0, 12.1, 12.3, and 13.3 kg) were used in seven scanning sessions on separate days, comprised of i) three baseline experiments in which [^{18}F]RX-2 was administered alone, ii) two receptor preblock experiments in two of the same monkeys used at baseline, in which SB 207710 (1.5 mg/kg, i.v) was administered

at 10 min before [^{18}F]RX-2 and in which the metabolite-corrected arterial input function was also measured, and iii) two challenge experiments in which SB 207710 (1 mg/kg, i.v.) was administered at 90 min after [^{18}F]RX-2 injection. In the two challenge experiments, the SB 207710 was administered as late as 90 min in order to acquire adequately long baseline data, but not necessarily to show the presence of specific binding as this was confirmed by the preblocking scans. Injected activities were 142–201 MBq. In the baseline and challenge experiments the specific activities of [^{18}F]RX-2 at time of injection were 46–114 GBq/ μmol .

Image analysis—PET Images were reconstructed using Fourier rebinning plus two-dimensional filtered back-projection. An averaged PET image was created by averaging all frames of PET images. Regional time–activity curves were generated from volumes of interest that were positioned on the monkey's MR image and then transferred to the averaged PET image. Monkey MR images of 0.5-mm contiguous slices were obtained using a 4.7-T MRI. A standardized monkey MRI template was then normalized to the monkey's MR image using SPM8 (Wellcome Trust Centre; London, UK). The monkey's MR image and the normalized MRI template were then coregistered to the averaged PET image. Volumes of interest from the template were then applied to the dynamic PET image to obtain regional decay-corrected time–activity curves. In one monkey, which did not have MRI, the PET images were directly coregistered to standardized monkey MR template. Uptake of radioactivity in each region of interest was expressed in SUV.

PET Data Analysis—Binding potential (BP_{ND}) was estimated with a simplified reference tissue model (SRTM) with 5-HT $_4$ receptor-poor cerebellum as reference tissue. Total distribution volumes (V_T) (Innis et al., 2007) were estimated for different regions with a one and two-tissue compartmental model applied to the acquired PET data and measured metabolite-corrected arterial input function. The temporal stabilities of V_T in cerebellum in the baseline and preblock experiments were assessed by estimating V_T from progressively time-truncated datasets. The identifiability of V_T was expressed as the standard error of V_T as a percentage of V_T itself; the lower the percentage the better the identifiability.

RESULTS

Pharmacology

RX-1 and RX-2 were found to have K_i values for human recombinant 5-HT $_4$ receptors of 1.1 and 0.8 nM, respectively (Table 1). In the same assay, SB 207710 was found to have a K_i value of 0.1 nM. RX-2 was found to have K_i values of $> 10 \mu\text{M}$ for a wide range of tested human recombinant receptors and binding sites (5-HT $_{1A}$, 5-HT $_{1B}$, 5-HT $_{1D}$, 5-HT $_{1E}$, 5-HT $_{2A}$, 5-HT $_{2B}$, 5-HT $_{2C}$, 5-HT $_3$, 5-HT $_{5A}$, 5-HT $_6$ and 5-HT $_7$, α_{1A} , α_{1B} , α_{1D} , α_{2A} , α_{2B} , β_1 , β_2 , β_3 , D $_1$, D $_2$, D $_4$, D $_5$, DAT, DOR, GABA, H $_1$, H $_4$, KOR, M $_1$, M $_2$, M $_4$, M $_5$, MOR, PBR, and SERT) and the rat brain benzodiazepine site. K_i values (nM) at other receptors and sites were 778 (α_{2C}), 3092 (D $_3$), 3103 (H $_3$), 1382 (M $_3$), 1402 (NET), 72 (σ_1), and 43 (σ_2).

Radiotracer Production

[^{11}C]RX-1 of greater than 99% radiochemical purity was produced in 25 and 34% average decay-corrected radiochemical yield from [^{11}C]iodomethane ($n = 3$) and [^{11}C]methyl triflate

($n = 7$), respectively, with specific activities in the range 57–154 GBq/ μ mol (average 103 GBq/ μ mol). [^{18}F]RX-2 of greater than 99% radiochemical purity was produced in 10% decay-corrected yield from [^{18}F]fluoride ion ($n = 9$) with specific activity in the range 57–150 GBq/ μ mol (average, 103 GBq/ μ mol). Each formulated radioligand was radiochemically stable for at least 2.5 h at room temperature.

Lipophilicity Determinations

The $\text{Log}D_{7.4}$ values measured for [^{11}C]RX-1 and [^{18}F]RX-2 at room temperature were 1.77 ± 0.01 ($n = 6$) and 1.54 ± 0.02 ($n = 6$), respectively.

Plasma Free fractions

The plasma free fractions (f_p) for [^{11}C]RX-1 and [^{18}F]RX-2 in monkey plasma that were inhibited with NaF at baseline were 0.442 ± 0.028 ($n = 6$) and 0.497 ± 0.030 ($n = 5$), respectively. Plasma free fraction increased slightly in preblock experiments.

Stability of [^{11}C]RX-1 in Buffer

[^{11}C]RX-1 and [^{18}F]RX-2 were $94.7 \pm 0.7\%$ ($n = 4$) $99.6 \pm 0.04\%$ ($n = 4$) stable, respectively, when incubated in phosphate buffer at room temperature up to 2.5 h (Table 2).

In vitro stability of [^{11}C]RX-1 in Whole Blood and Plasma

The extraction of radioactivity into acetonitrile from whole blood or plasma that had been incubated with [^{11}C]RX-1 was in all cases greater than 85%, and for incubate containing sodium fluoride plus PMSF extraction was 91.9% (Table 1). After 30 minutes incubation of [^{11}C]RX-1 in whole blood or plasma, [^{11}C]RX-1 was 16.8% and 20.0% changed, respectively. Even after 5 min incubation 3–5% degradation occurred. Therefore it was necessary to stabilize the radioligand in blood in vitro for accurate radiometabolite analyses. Inclusion of putative esterase inhibitor(s) (Fahrney and Gold, 1963) in the incubates reduced hydrolysis considerably. The combined use of sodium fluoride and PMSF was especially effective (Table 1). Sodium azide alone was ineffective in stabilizing [^{11}C]RX-1 in blood. PMSF, being lipophilic, is expected to be able to cross blood cell membranes and inhibit esterases inside the cell. Based on these results, we chose the combined use of sodium fluoride and PMSF to inhibit ester hydrolysis during the processing of monkey blood samples for radiometabolite analysis. The final protocol for inhibiting esterase activity in collected blood samples was to draw the blood sample (1 mL) into a syringe containing aqueous NaF solution (10 μ L). This was then dispensed into a heparin-coated Eppendorf tube (1.5-mL) containing a solution of PMSF in DMSO (5 μ L) plus saline (5 μ L), and mixed well.

Analysis of radiometabolites in plasma

For [^{11}C]RX-1 and [^{18}F]RX-2, the extraction efficiencies of all samples were $95 \pm 6\%$, ($n = 74$) and $95 \pm 2\%$ ($n = 36$), respectively. During each monkey study, whole blood and plasma standards were constructed to monitor the effectiveness of esterase inhibition. For [^{11}C]RX-1, inhibition of hydrolysis in whole blood was $94 \pm 2\%$ ($n = 5$) and in plasma $96 \pm 3\%$ ($n = 5$), and for [^{18}F]RX-2 inhibition in whole blood was $93 \pm 2\%$ ($n = 2$) and in

plasma $94 \pm 2\%$ ($n = 2$). Each radioligand was found to be rapidly metabolized in vivo such that radiometabolites represented 50% of the radioactivity in plasma within 5–7 min. [^{11}C]RX-1 gave one major radiometabolite and [^{18}F]RX-2 gave two major unresolved radiometabolites. These radiometabolites eluted in or near the void volume, and were well resolved from parent radioligands (Figure 2).

Monkey PET Experiments

Upon injection of [^{11}C]RX-1 or [^{18}F]RX-2 into monkey at baseline, radioactivity rapidly accumulated in whole brain and on average peaked at 2.3 SUV after 5 min and 3.1 SUV after 6 min, respectively. Peak radioactivity uptakes then smoothly declined at rates that ranged from slowest in 5-HT₄ receptor-rich putamen to fastest in 5-HT₄ receptor-poor cerebellum (Figure 3). For each radioligand, the summed PET image from 10 min after injection showed a distribution of radioactivity reflecting the expected distribution of 5-HT₄ receptors (Figure 4). In receptor preblock experiments, where the high-affinity selective 5-HT₄ antagonist SB 207710 (Kaumann et al., 1994; McClean and Coupar 1994) was administered before radioligand, brain radioactivity uptake was again rapid, reaching an early peak. Subsequent decline in radioactivity level was much more rapid than in the baseline experiments with the same radioligand (Figure 3). Peak brain radioactivity levels were higher than in baseline experiments with [^{11}C]RX-1, but lower with [^{18}F]RX-2. In these preblock experiments, summed images from 10 min after radioligand injection showed a low uniform distribution of radioactivity (Figure 4). In experiments where SB 207710 was administered at 90 min after [^{18}F]RX-2 injection, radioactivity levels in all regions rapidly fell to that in cerebellum (data not shown). The fluoromethoxy group of [^{18}F]RX-2 was dideuterated as a precaution against excessive radiodefluorination. PET experiments with [^{18}F]RX-2 showed no appreciable accumulation of radioactivity in skull.

For both [^{11}C]RX-1 and [^{18}F]RX-2 the rank order of regional BP_{ND} values determined using a simplified reference tissue model with cerebellum as reference region were in the rank order striatum > hippocampus > frontal cortex > thalamus > midbrain. Values were slightly but not significantly higher (~20%) for [^{18}F]RX-2 than for [^{11}C]RX-1 across all regions (Table 3).

For both [^{11}C]RX-1 and [^{18}F]RX-2, a one-tissue compartment model gave good fits to the PET whole brain data in both baseline and preblock experiments (Figure 5). The two-tissue compartment model did not converge for nearly half of the brain regions for all scans and distribution volume (V_T) was poorly identified for some regions where fits converged. Regional V_T values were in the rank order caudate > putamen > hippocampus > cerebellum (Figure 5). Values were greater for [^{18}F]RX-2 than for [^{11}C]RX-1. Furthermore, both [^{11}C]RX-1 and [^{18}F]RX-2 showed a strong positive correlation between V_T and BP_{ND} values for all regions with Pearson R values of 0.90 and 0.93 respectively (data not shown). For each radioligand in preblock experiments, V_T values were substantially reduced in caudate, putamen and hippocampus to a very similar level that was still somewhat higher than that in cerebellum (Figure 6). From these data it is estimated that 5-HT₄ receptor-specific binding represented 50–60% of total radioligand binding in striatum. For [^{11}C]RX-1, the V_T values in cerebellum were similar in both baseline and preblock

indicating no specific binding to 5-HT₄ receptor-poor cerebellum. However, for [¹⁸F]RX-2, the V_T values in cerebellum were slightly higher at baseline than in the preblocked condition, indicating some level of specific binding in cerebellum.

For both [¹¹C]RX-1 and [¹⁸F]RX-2, V_T rapidly reached stable values in receptor-poor cerebellum, namely within 5% of the stable value by about 50 and 25 min for [¹¹C]RX-1 and [¹⁸F]RX-2, respectively (Figure 7). Identifiability of V_T between 30 and 120 min was good at < 5 and < 3% for [¹¹C]RX-1 and [¹⁸F]RX-2, respectively.

DISCUSSION

The two new PET radioligands for 5-HT₄ receptors, [¹¹C]RX-1 and [¹⁸F]RX-2, are analogs of SB 207710 and are quite structurally similar to recently developed [¹¹C]SB 207145 (Kornum et al., 2009), [¹⁸F]MNI-698 (Callié et al., 2013), and [¹⁸F]MNI-699 (Callié et al., 2013) (Figure 1). They are all potentially susceptible to rapid ester hydrolysis in vivo and in blood in vitro, as we observed for [¹¹C]RX-1 and [¹⁸F]RX-2 in this study. Unlike their predecessors that may generate a brain-penetrating radioactive alcohol as a radiometabolite, [¹¹C]RX-1 and [¹⁸F]RX-2 have the radiolabel on the other side of the ester group (Xu et al., 2010). After ester hydrolysis, the radiolabeled RX ligands are each expected to generate a polar carboxylic acid as radiometabolite, which should be less susceptible to penetrate the blood–brain barrier than the parent radioligand, and thus enable accurate quantification of 5-HT₄ receptors in vivo (Pike, 2009). In the present study, we investigated [¹¹C]RX-1 and [¹⁸F]RX-2 for in vivo imaging and quantification of 5-HT₄ receptors in rhesus monkey brain. Both radioligands showed high average whole brain uptake (peak SUV >2) consistent with their measured moderate lipophilicity (*clogD*) values (Waterhouse, 2005; Pike, 2009). Regional radioactivity uptake decreased in the order striatum > cortex > cerebellum, which is consistent with the known distribution of 5-HT₄ receptors in rhesus monkey brain (Caillé et al., 2013). With cerebellum as reference region, the baseline BP_{ND} was slightly but not significantly higher (~20%) for [¹⁸F]RX-2 than for [¹¹C]RX-1 across regions. Binding to striatum was 50–60% specific with stable and well-identified V_T from 60 min brain data for both radioligands.

At baseline, for both [¹¹C]RX-1 and [¹⁸F]RX-2, the peak uptakes in the striatum were high (4–7 SUV) and similar, but the washouts were rapid (>50% decline to that of peak in 60 min from injection). In comparison, [¹¹C]SB 207145 and [¹⁸F]MNI, when tested in minipig (Kornum et al., 2009) and rhesus monkey (Caillé et al., 2013), respectively, had similar peak uptakes but slower washout (<15% decline to that of peak in 60 min) in striatum. The rapid washout of RX radioligands indicates reversible binding which could facilitate quantification from imaging data acquired over a shorter time span.

The baseline BP_{ND} values in striatum for [¹¹C]RX-1 was 1.7 to 3.3-fold lower, and for [¹⁸F]RX-2 was 1.4 to 2.8-fold lower than that for [¹¹C]SB 207145, which was evaluated in minipig (Kornum et al., 2009) and human (Marner et al., 2009) brain. The BP_{ND} values for [¹⁸F]MNI ligands are not available, but judging from the reported target-to-cerebellar SUV ratios (Callié et al., 2013), BP_{ND} may be estimated to be 3 to 4 fold higher for the MNI than for RX radioligands. Such variations could be due to affinity differences between the ligands

as well as species differences, but it does indicate that RX radioligands have a lower proportion of specific binding than their counterparts.

To quantify the 5-HT₄ receptor density in terms of distribution volume, we used brain PET data and metabolite-corrected arterial input function, and did kinetic modeling using one-tissue and two-tissue compartment models. The procedure we developed for inhibiting esterase action on RX radioligands proved effective for making accurate measures of unchanged radioligand in monkey arterial plasma samples. A one-tissue compartment model provided better fits in all scans for both RX radioligands, suggesting that kinetic exchange rates between the specific and non-displaceable compartments are fast and indistinguishable. Similarly, kinetic modeling of [¹¹C]SB 207145 showed a one-tissue model to be superior to a two-tissue model in minipig brain (Kornum et al. 2009). However, the modeling result may vary depending on species, because two-tissue modeling has been found superior to one-tissue modeling for [¹¹C]SB 207145 in human brain (Marner et al., 2009).

Both RX radioligands showed stable values of distribution volume even at 60 min of scan data with less than 5% change in V_T and its identifiability in cerebellum, a receptor-poor region that is prone for accumulation of radiometabolite in the absence of specific binding. The stable V_T values indicated negligible accumulation of radiometabolites in brain and suggested that 60 min of dynamic scan data are adequate for accurate quantification of 5-HT₄ receptors in monkey brain. Among reported 5-HT₄ radioligands, only [¹¹C]SB 207145 has been well characterized for time stability of distribution volume, and this showed larger bias and variance for acquisitions less than 100 min in humans (Marner et al., 2009). The time stability of [¹⁸F]MNI-698 and [¹⁸F]MNI-699 ligands indirectly determined in rhesus monkeys by SUV ratios of target region to cerebellum resulted in stable values at 80 min and 160 min post injection, respectively (Caillé et al., 2013). In comparison to these ligands, the design of RX ligands that likely generates hydrophilic carboxylic acid as radiometabolites following in vivo ester hydrolysis may have contributed to the better time stability of distribution volume.

Both [¹¹C]RX-1 and [¹⁸F]RX-2 showed a modest 50–60% of total binding in striatum to be receptor-specific. However, [¹⁸F]RX-2 unexpectedly showed specific binding in cerebellum with slightly higher V_T values under baseline than under preblocked condition (Figure 6). Because free fraction of radioligand in plasma may influence the binding, V_T was corrected for f_p , but still some specific binding by [¹⁸F]RX-2 was found in cerebellum (data not shown). In primates, cerebellum is generally considered to be a 5-HT₄ receptor-poor region from various in vitro and in vivo studies using multiple 5-HT₄ specific ligands (Reynolds et al., 1995; Varnas et al., 2003; Marner et al. 2009; Caillé et al. 2013). Specific binding to 5-HT₄ in cerebellum is thus unexpected, but this may be possible if the radioligand and the blocking agent are not fully selective to 5-HT₄ receptor. Some 5-HT₄ ligands have shown binding to σ_1 and σ_2 receptors (Bockaert et al., 2004). In fact, [¹⁸F]RX-2 showed ~2-fold higher affinity to σ_1 and σ_2 receptors than [¹¹C]RX-1 (K_i of 72 and 43 nM vs 211 and 148 nM to σ_1 and σ_2 receptors, respectively). The σ_1 and σ_2 receptor density in striatum is however low (Abate et al., 2013, James et al., 2014) and thus may minimally influence binding of [¹¹C]RX-1 and [¹⁸F]RX-2.

In receptor pre-block experiments, both [^{11}C]RX-1 and [^{18}F]RX-2 showed some residual binding in extra-cerebellar regions (Figure 6) indicating somewhat higher levels of non-displaceable binding. The blocking agent may not have blocked all 5-HT₄ receptors, but could not be given safely at higher dose. However, the blocking dose was high enough that binding became relatively uniform across brain from high to low density regions. Furthermore, non-displaceable binding might also be due to non-selective binding, and perhaps more so for [^{18}F]RX-2 which showed some specific binding in cerebellum.

In summary, both [^{11}C]RX-1 and [^{18}F]RX-2 showed suitable in vivo characteristics to measure 5-HT₄ receptors in monkey brain. The brain uptake was high, quantifiable by kinetic modeling as distribution volume that was well-identified even with 60 min scan duration. Importantly, distribution volume was found to be stable over shorter scan duration indicating negligible radiometabolite entry to brain and thus validating the radioligand design strategy for the RX radioligands. The application of these radioligands to measure human 5-HT₄ is warranted, but arguable in light of the other currently available radioligands that may have better characteristics.

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$[^{123}\text{I}]$ SB 207710: $\text{X} = ^{123}\text{I}$, $\text{R} = ^n\text{Bu}$

$[^{11}\text{C}]$ SB 207145: $\text{X} = \text{Cl}$, $\text{R} = ^{11}\text{CH}_3$

$[^{18}\text{F}]$ MNI-698: $\text{X} = \text{Cl}$, $\text{R} = \text{CH}_2\text{CH}_2^{18}\text{F}$

$[^{18}\text{F}]$ MNI-699: $\text{X} = \text{Cl}$, $\text{R} = \text{CH}_2\text{CH}_2\text{CH}_2^{18}\text{F}$

$[^{11}\text{C}]$ RX-1: $\text{R} = ^{11}\text{CH}_3$

$[^{18}\text{F}]$ RX-2: $\text{R} = \text{CD}_2^{18}\text{F}$

Figure 1.
Structures of 5-HT₄ receptor radioligands.

Figure 2.

(A) Radiochromatogram from a 3.02-min plasma sample collected after intravenous injection of [^{11}C]RX-1 (195 MBq) into a rhesus monkey. At least two unresolved radiometabolites were detected near the void volume. These radiometabolites were well resolved from the more lipophilic parent radioligand, which eluted at 3.8 min (11.4 mL) and which represented 48.33% of sample radioactivity. (B) Radiochromatogram of a 3.02-min plasma sample collected after intravenous injection of [^{18}F]RX-2 (196 MBq) into a rhesus monkey. Again, at least two unresolved radiometabolites were detected near the void

volume. These radiometabolites were well resolved from the parent radioligand, which eluted at 3.9 min (17.6 mL) and which represented 60.5% of sample radioactivity.

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Figure 3.
Time course of radioactivity concentrations in brains of rhesus monkeys after intravenous injection of $[^{11}\text{C}]\text{RX-1}$ and $[^{18}\text{F}]\text{RX-2}$.

Figure 4.

Brain PET images at the level of striatum averaged from 10 min to end of scan in rhesus monkeys after injection of $[^{11}\text{C}]\text{RX-1}$ and $[^{18}\text{F}]\text{RX-2}$ at baseline and after preblock with SB 207710 (1 mg/kg for $[^{11}\text{C}]\text{RX-1}$ and 1.5 mg/kg for $[^{18}\text{F}]\text{RX-2}$ at 10 min before radioligand injection). The corresponding MR image slices are shown to the left of PET images.

Figure 5.

Brain putamen time-activity curves in rhesus monkeys after injection of [^{11}C]RX-1 (left panel) and [^{18}F]RX-2 (right panel) at baseline and after preblock with SB 207710 (1 mg/kg for [^{11}C]RX-1 and 1.5 mg/kg for [^{18}F]RX-2 at 10 min before radioligand injection). The lines in the brain curves represent fitting of measured data by one-tissue compartmental modeling.

Figure 6. Distribution volume (V_T) values under baseline and preblocked conditions for $[^{11}\text{C}]\text{RX-1}$ and $[^{18}\text{F}]\text{RX-2}$. Data represent mean $\pm$ SD values from two rhesus monkeys for each radioligand. CAUD = Caudate; PUT = Putamen; HIPP = Hippocampus; CBL = Cerebellum

Figure 7.

Distribution volume (V_T) values and their identifiabilities (% SE) as a function of duration of image acquisition at baseline and after receptor blockade with SB 207710 in rhesus monkeys. V_T was calculated for cerebellum using a one-tissue compartment model. Data represent mean values from two rhesus monkeys for each radioligand.

Table 1Binding affinities of 5-HT₄ PET ligands

Ligand	h5-HT ₄ K _i (nM) ^a	p5-HT ₄ K _d (nM) ^b	r5-HT ₄ K _i (nM) ^c
SB207710	0.1		
SB207145		0.39 ± 0.06	
RX-1	1.1		
RX-2	0.8		
MNI698			0.20 ± 0.05
MNI699			0.07 ± 0.01

^aFor human recombinant 5-HT₄ receptors (measured by NIMH-PDSP).^bFor Göttingen minipig striatal membranes (Kornum et al., 2009).^cFor rat striatal membranes (Callié et al., 2013).

Table 2

The effect of enzyme inhibitors on the in vitro stability of [^{11}C]RX-1 in whole blood after its incubation for 30 min

Incubation medium	Final inhibitor Concentration (mM)	Radioactivity recovered into acetonitrile (%)	Stability [†] (%)
Phosphate buffer		95.9	99.5
Untreated whole blood		91.1	73.2
Untreated plasma		97.2	80.0
Whole blood + NaN ₃	64	84.9	76.9
Whole blood + NaF	9.5	89.7	99.3
Whole blood + NaF + PMSF	9.5 + 10	91.9	99.6

[†]Stability = % parent in sample/radiochemical purity of [^{11}C]RX-1.

Table 3

Regional BP_{ND} values by SRTM with cerebellum as reference region for [^{11}C]RX-1 and [^{18}F]RX-2

Regions	Binding potential (BP_{ND})	
	[^{11}C]RX-1	[^{18}F]RX-2
Striatum	1.02 ± 0.03	1.19 ± 0.15
Hippocampus	0.48 ± 0.06	0.58 ± 0.12
Frontal Cortex	0.31 ± 0.10	0.43 ± 0.13
Thalamus	0.31 ± 0.07	0.38 ± 0.09
Midbrain	0.23 ± 0.06	0.28 ± 0.13

Data represent mean $\pm$ SD from $n = 3$ for [^{11}C]RX-1 and from $n = 5$ for [^{18}F]RX-2.